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OPTIMIZING STABILITY AND BIOAVAILABILITY OF ACTIVE INGREDIENTS FOR TOPICAL FORMULATION

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ABBREVATION

AAPH	:	2,2-Azobis(2-Methylproprionamide)
AB	:	Avobenzone
ACL	:	Antioxidant Capable Liposoluble
ACW	:	Antioxidant Capable Watersoluble
B1	:	Synthetic derivative of Baicalin
BZD	:	Benzydamine Hydrochloride
$CHCl_3$	:	Chloroform
DMAP	:	4-Dimethylaminopyridine
DMSO	:	Dimethylsulfoxide
DPPH	:	2,2-Diphenyl-1-picryl Hydrazyl
DT	:	Dissolution Test
eNOS	:	Endothelial Nitric Oxide Synthetase
Esp1-Esp7	:	Synthetic derivatives of Hesperidin
EtOH	:	Ethanol
FDA	:	Food and Drug Administration
FRAP	:	Ferric Reducing Antioxidant Power
GLA	:	Glycirrethic Acid
H_2O_2	:	Hydrogen Peroxide
IBU	:	Ibuprofen
iNOS	:	Inducible Nitric Oxide Syntethase
K_2CO_3	:	Potassium Carbonated
MeOH	:	Methanol
$NaHSO_3$	:	Sodium Bisulfite
nNOS	:	Neuronal Nitric Oxide Synthetase
NO	:	Nitric Oxide
NP	:	NanoParticles
OMC	:	Octyl Methoxy Cinnamate
ORAC	:	Oxygen radical absorbance capacity
PBS	:	Phosphate Buffered Saline
PBSA	:	2-Phenyl-1H-Benzymidazole-Sulfonic-Acid
PLC	:	Photochemiluminescence
PS	:	Polystyrene
RNS	:	Reactive Nitrogen Species
ROS	:	Reactive Oxigen Species
SA1	:	Synthetic derivative of Salicin
SAD	:	Seasonal Affective Disorder
SDA	:	Sabouraud Dextrose Agar
SPF	:	Sun Protection Factor
TPTZ	:	2,4,6-Tripyridyl-Triazine
UVAPF	:	UVA Protection Factor

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1. INTRODUCTION

1.1 Drugs and Cosmetics

According to AIFA *"A drug is a substance or group of substances used to cure or prevent a specific disease"* while a cosmetic is "*any substance or mixture intended to be applied on the external surfaces of human body (skin, hair, nails, lips and external genital organs) or on the teeth and the mucous membranes of the oral cavity with the purpose, exclusively or mainly, to cleaning, perfuming them, changing their appearance, protecting, keeping in good condition or correcting body odors"* [1].

The rule of cosmetics is very clear according to the law: a cosmetic must be different from a drug, it has not therapeutic purpose and not boasts of therapeutic activities.

Although, the difference between drugs and cosmetics is defined, it is not always easy to recognize one from the other, especially because the pharmaceutical field, in recent years, is gradually getting closer to cosmetics.

In fact, in the past, there was a predominance of the use of synthetic active principles, mainly for systemic use and sometimes for local use: the few pharmaceutical products for topical use had the only function to be active, and there was not a research to a more "pleasant formulation". In recent years, the trend has reversed; when it is possible, systemic formulations are not being used so much any more, while active ingredients of natural origin are to be preferred for local and topical application which make them not only functional but also enjoyable for people. In this way, pharmaceutical products will look like cosmetics.

On the other hand, since the late 90s, the gradual appearance of new technologies and the development of cosmetic formulations containing new active molecules, led to the creation of more innovative cosmetics compared to the traditional ones [2].

However, it is true that some cosmetics are borderline products with the ability to prevent diseases. It is what happens when a product has two different uses. For example, creams with sunscreens: SPF in a cream prevents diseases (drug use) while a normal moisturizing cream is a product that is applied on the skin to improve it (cosmetic use); in fact sunscreens are not cosmetic products according to FDA guidelines [3].

Even an active ingredient, commonly used for cosmetic purposes, such as Vitamin C (principally for its antioxidant activity) is used in combination with drugs in order to increase the immune system or as a coadjuvant in colds. Another example is for acne products, where many medications can increase their effectiveness by using a cosmetic cream with astringent, purifying and stabilizing activity on the hydro-lipidic film.

Therefore, it can be confirmed that the correct classification of a cosmetic product must be carried out with a so called "case by case" approach that considers all the product features: composition, shape, indications on the label.

1.2 Polyphenols and Oxidative stress

Natural polyphenols are the biggest group of antioxidant compounds in human diet. This class of molecules, which presents more than 8000 structural variants, includes various secondary metabolites of plants characterized by the presence of aromatic rings with one or more phenolic functionality.

They are generally divided into different groups according to the number of aromatic rings and structural elements linked to them:

- Phenolic acids which includes Benzoic acids (such as Gallic acid), Hydroxycinnamic acids (like Caffeic acid), Ferulic or coumaric acid;
- Flavonoids;
- Not-flavonoid polyphenols such as trans-resveratrol.

The structural feature responsible for the antioxidant and radical-scavenger activity of these derivatives is precisely the presence of a phenolic group. These compounds are able to give their hydrogen atom of phenol to free radicals and stop the propagation of the typical chain reactions of oxidative processes. The presence of a second hydroxyl group in ortho position, typical of catechol structures, leads to a reduction in enthalpy of the O-H bond dissociation in order to further facilitate radical reactions [4]. The effectiveness of

these compounds is also due to their ability to delocalize the unpaired electrons of the phenolic radicals that have being formed and the chelation of transition metals [5].

The information on the absorption, distribution and metabolism of these molecules is still incomplete. Many polyphenols are absorbed by the intestine in their original form or in modified forms. For example, some esterified or glycosylated compounds cannot be absorbed in their original form, but must be hydrolyzed by the intestinal enzymes or by the bacteria in the colon. Such molecules are subsequently metabolized giving rise to products that maintain only part of the antioxidant capacity of the initial compounds and finally excreted.

In any case, the polyphenols which are not absorbed remain throughout the digestive tract where they can do its radical-scavenger action against several free radicals present in the gastric compartment [6].

A lot of polyphenols have also different physiological activities, like antiviral, antifungal and antibacterial activity [7] (Figure 1).

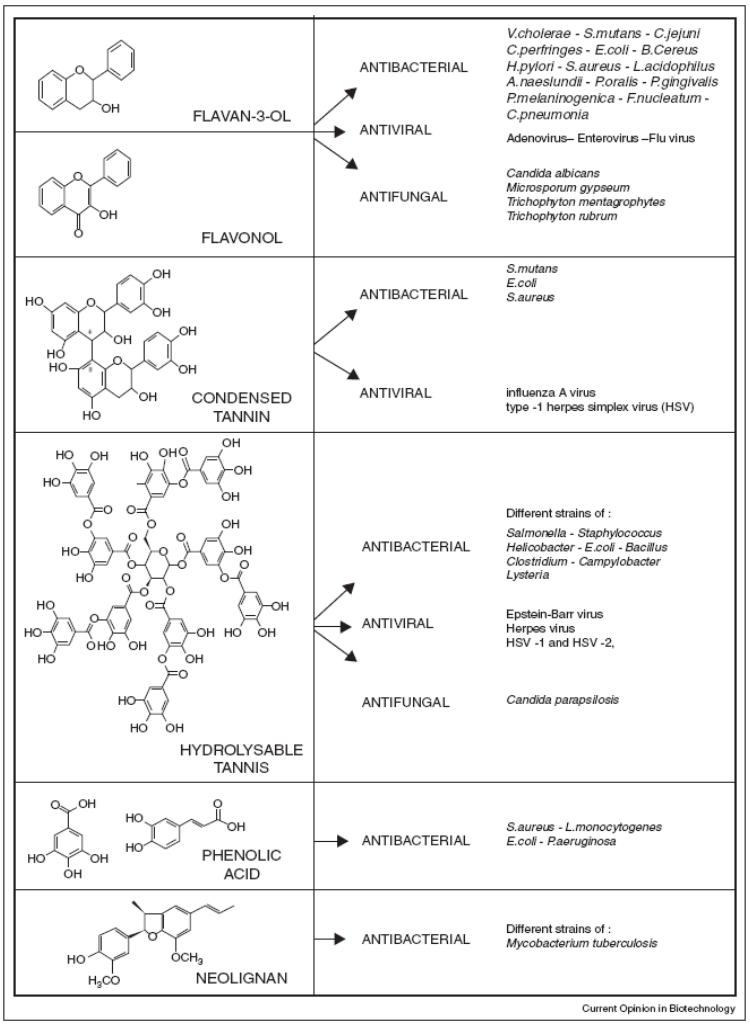


Figure 1. *Therapeutic active of polyphenols.*

Oxidative stress is a direct result of the damaging action exercised by an extremely high amount of free radicals on cells and tissues of our body (Figure 2) [8]. Free radicals are atoms or groups of atoms capable of reacting with organic macromolecules present in the cell, leading to harmful consequences as functional and structural abnormalities that result in cell death. The damage is due to the fact that agents are very reactive and stabilized, stripping electrons from molecules which they come in contact with or by binding themselves to other free radicals.

Figure 2. *Oxidative stress damages.* http://www.geneactivatornrf2.org/what-is-oxidative-stress/#.WI8MX1PhDIU

A proportion of free radicals is also produced in normal conditions due to the cell metabolism and it is naturally neutralized by the endogenous antioxidant defenses. However, excessive levels of these molecules, are not beneficial and constitute a serious threat to the integrity of the cells. The dangerous radicals are classified into reactive oxygen species, commonly known as **ROS** (Reactive Oxygen Species) and into reactive nitrogen species, **RNS** (Reactive Nitrogen Species). Among the latter, the most interesting compounds are Nitric Oxide (NO) and peroxynitrite; NO is produced by the enzyme NO synthase of which there exist three types in humans: neuronal NO synthase (nNOS) are present in neurons and skeletal muscles, inducible NO synthase (iNOS) in the cardiovascular system and in the cells of the immune system, and endothelial NO synthase (eNOS) present in the endothelium. Nitric oxide is a neurotransmitter involved in the immune response and a potent vasodilator. On the other hand, peroxynitrite (ONOO-) can damage lipids, proteins and DNA, and it is produced by the reaction between nitric oxide and superoxide anion, and then it is converted into HNO_2 by enzymes present in the cytosol and in mitochondria. **ROS** are free radicals and they are broken down into oxygen free radicals such as superoxide anion (O^{2-}), hydroxyl radical (• OH) and hydrogen peroxide (H_2O_2).

Most of the superoxide anions produced at a cellular level derive from the defense mechanisms that the inflammatory process applies in respect of potentially harmful micro-organisms [9].

Both **ROS** and **RNS** production can result from external or internal causes; external causes can be certain physical agents (for example Ultraviolet radiation), several chemicals (like hydrocarbons, herbicides, food contaminants, drugs) and some infectious agents (viruses and bacteria), while internal causes are due to an accelerated metabolism (for example, after intense and prolonged physical exertion, without proper training) or numerous diseases (obesity, diabetes etc.).

When the body is healthy it is able to prevent the damage from free radicals thanks to the defense systems (antioxidants) which may be endogenous [10], ie produced within the body, or exogenous if supplied with the diet or by the use of supplements in various formulations. Endogenous antioxidants are classified into enzymatic (or primary) and non-enzymatic (or secondary).

Therefore, in our body there is a delicate balance between production (external or internal) and the amount of free radicals which causes the breakage of the cellular injury that, if severe and prolonged in time, leads to an acceleration of the aging process and the onset of many degenerative diseases [11]. For this reason, over the years researchers have given considerable importance to the research of molecules that have an antioxidant capacity.

Nature is full of compounds which have this activity accompanied with several other therapeutic capabilities, but their use is often difficult because polyphenols are usually hard to formulate because poorly soluble, unstable to light and pH changes [12].

1.3 UV filters and Sunlight

The sun produces a huge amount of radiation at different wavelengths, including cosmic rays, gamma rays, X-rays, UVB and UVA radiation, visible radiation and infrared radiation. All high-energy cosmic, gamma and X-rays are reflected or absorbed by the atmosphere that surrounds our planet. Most of the UV radiation is efficiently absorbed by the stratospheric ozone layer [13].

All UVC (200-280 nm) radiation is efficiently absorbed by the ozone layer and none of them reaches the surface of the earth. In addition, most of the UVB and UVA radiation is absorbed by the ozone layer.

Although UVA and UVB rays are only a small portion of the radiation that reaches the earth surface, they are the most dangerous to human health, in particular for organs such as skin, eyes and hair, not only because they are directly irradiated, but also for the presence of molecules in tissues containing chromophores capable of absorbing the radiation, such as melanin, DNA, RNA, proteins, lipids and amino acids (tryptophan and tyrosine) [14].

According to Plank's law there is an inverse relationship between wavelength and energy, in fact lower wavelengths of radiation have higher energy:

$$E = h * v = \frac{h * c}{h}$$

Equation 1. *Plank's law.*

Where,

h is Plank's constant,

v is the frequency of electromagnetic radiation;

c is the speed of light.

So, the lower is the wavelength the greater the energy of radiation will be.

Although the intensity can be changed by factors such as latitude (at the equator, the ozone filters layer is naturally thinner and the radiation travels a shorter distance before reaching the Earth, and in this way it is more intense), altitude (the further from the sea level you go the atmosphere progressively becomes thinner, so the radiation intensity is increased) and agents in the atmosphere (fog, clouds or pollution) [15].

Studies on human keratinocyte cultures exposed to UVA and UVB rays, have shown a marked increase in the expression and production of beta-endorphin only in the cells exposed to UVA radiation [16]. Even skin biopsies of adults exposed to UVB radiation have shown increased expression of beta-endorphin in keratinocytes [17].

Beta-endorphin, an endogenous opioid peptide, has shown to improve not only the well being of a person, but it can also produce pain relief and relaxation. Sunlight deprivation is associated with depression. In winter, this can cause seasonal affective disorder (SAD) in people who are sensitive.

Our circadian rhythm is controlled by the blue light that is absorbed by the photoreceptors in the eyes and this slows the production of melatonin [18]. For some people the decrease in the intensity of sunlight due to seasonal variations prevents the melatonin suppression and this induces the person to sleep more and may become listless and depressed [18].

On the other hand, exposure to sunlight can cause very serious damage to our body, acute and chronic manifestations of different types in the skin, such as tanning, erythema, thickening, photoaging, but also immunosuppression and mutations, responsible for photocarcinogenesis [20].

In particular, UVB rays have higher energy and are more cytotoxic and mutagenic, while UVA rays are able to penetrate into the deeper layers of the skin and they are responsible for an indirect damage through the formation of reactive oxygen species [21].

When the skin is exposed to sun radiation, it uses tanning as a photo protection mechanism and the melanin produced can absorb much of the energy of the radiation and then remit it into the form of another energy [22].

Prolonged exposure, however, makes this defense mechanism ineffective.

One of the methods to increase the natural defense of the skin is to apply creams or lotions containing sunscreens on the skin.

The available sunscreens on the market today, are divided into Chemical Fliters and Physical Filters.

Titanium dioxide and zinc oxide are physical filters that work by creating a barrier on the skin and capable of reflecting visible light and ultraviolet rays.

The organic filters, instead, act as melanin, absorbing the ultraviolet radiation: electrons go to an excited state then they return to the fundamental state, re-emitting energy in the form of a lower energy radiation.

UV filters are divided into UVB, UVA or broad-spectrum rays (UVA and UVB).

The UVB filters are among the most efficient, blocking up to 90% of the radiation. However, sunscreens normally contain a combination of organic filters and inorganic UV filters, which ensure an effective protection across the whole UVA and UVB range [23].

According to the European Cosmetics Regulation No. 1223/2009 [24] molecules used as a filter should be photo and chemically stable and inert to heat and to other cosmetic ingredients. In addition, they should not exhibit cytotoxicity or photo toxicity, or penetrate the stratum corneum, remaining on the surface layers of the skin [25].

A single molecule rarely manages to satisfy all these requirements at the same time, and in order to remedy the problem the use of a single UV filter is not enough, but a combination of active ingredients is necessary[26].

The benzymidazole ring appears in many sunscreens. One of the most well-known chemical filters that contain it is 2-phenyl-1H-benzimidazole-5-sulfonic acid (PBSA), widely used in cosmetic formulations as sunscreen because it has a strong absorption in the region of UVB, between 290 and 320 nm. Being soluble in water makes its use in cosmetic formulations easier, especially in the combination with lipophilic filters, thus allowing greater protection thanks to a synergistic effect. PBSA is considered efficient and secure in preventing rashes, because very few events of irritation and sensitization, phototoxicity and allergy have been registered [27].

1.4 Human skin

The largest organ of our body is the skin, in fact, it covers the surface of our body and it also continues inside it with the mucous membrane.

The main function of the skin is to protect the organism from the external environment, adjust the secretions and maintain the body temperature.

Anatomically, the skin is composed of three different layers (Figure 3):

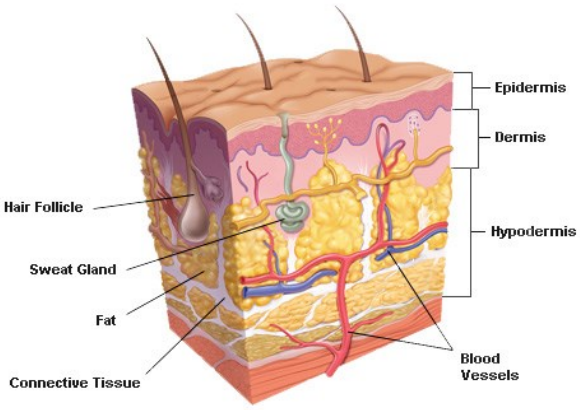


Figure 3. *Human skin*. (© 2014 WebMD, LLC).

Epidermis acts as a barrier: on the one hand it prevents the penetration of water, extraneous substances and microorganisms and, on the other hand, it hinders the loss of water and electrolytes from the body.

It is a thick tissue, about 0.2 mm, formed by several layers. From the anatomical point of view, it is a stratified squamous epithelium and consists of different natural cells and keratinocytes are the predominant cells of the epidermis, about 90%. These cells, after undergoing a specific differentiation process, are transformed into flattened cells without core, called corneocytes. The remaining cells are melanocytes which synthesize melanin, Langerhan's cells which have immunological functions and Merkel cells, which act as mechanoreceptors.

The epidermis can be summarized into four different layers from the innermost to the outermost: the germinal layer, the spinous layer, the granular layer and the horny layer. In some areas of the body (the palmoplantar region) an additional layer, the stratum lucidum, can be seen between the granular and horny layers.

Derma is immediately below epidermis, characterized by the presence of cells such as fibroblasts, mast cells and dermal dendrocytes.

Derma is a very elastic tissue and highly innervated, vascularized and characterized by a matrix with great capacity to retain water, polysaccharides and proteins. The main proteins that are present are elastin and collagen.

Hypodermis is the deepest skin layer, mainly composed of adipocytes, and it plays an important role in thermoregulation, insulation, energy supply (nutritional deposit) and protection from mechanical injury.

In hypodermis, follicles and sweat glands are formed: this is where they receive nourishment and surrender their waste products.

1.5 Skin and drugs

Creating topical drugs is quite complicated to obtain because the skin acts as a protective barrier of our organism [28].

Drugs can be absorbed in different ways and it depends on the chemical nature of them: through the glandular annexes (pilosebaceous apparatus and eccrine glands active to suitable lipophilic and hydrophilic molecules respectively) or through transepidermal way (intercellular and / or transcellular) [29].

Of the latter, the intercellular way is used by compounds with high molecular weight (MW> 2000 Da). In this way, drugs can not be metabolized because most of the enzymes are located within the cell. The direct transcellular penetration, however, consists in the passage of the molecules through the cell membranes of corneocytes, which due to their structural characteristics and this is one of the main obstacles [30]. Subsequently, the polar molecules migrate to the aqueous areas of the corneocytes while the lypophilic molecules move towards areas with greater lipid content. It is clear that the whole process is based on a passive diffusion. The transcellular passage is easier for molecules which have a low molecular weight (MW <2000 Da) and which are "correctly lipid soluble" [31]. The process of crossing the stratum corneum can be represented by Fick's law, assuming that it behaves like a membrane, not caring about all the biopharmaceutical and physical-chemical factors that influence this process.

$$\frac{dq}{dt} = \frac{R * Ds * A * Cs}{h}$$

Equation 2. *Fick's law.*

Where:

Cv: concentration of the active ingredient of the vehicle;

R: coefficient of distribution of the active ingredient between vehicle and barrier;

Ds: diffusion coefficient of the active substance through the epidermis;

A: area affected absorption;

H: thickness crossed.

This law shows that the transport across the stratum corneum is directly proportional to the solubility and diffusibility of the substance applied. The relative solubility of a solute is expressed by the partition coefficient skin/vehicle and it refers to the ratio between the solubility of the substance not only in the stratum corneum but also in the vehicle. Therefore, this parameter indicates the affinity of a drug to the stratum corneum and its ability to separate from the vehicle [32].

Hence, the skin is a target which is not easy to use but for its physical properties it is a storage site for a large number of drugs; nevertheless, when some drugs are absorbed by the skin they last longer and allow a single daily application.

It is important to distinguish between transdermal and topical formulations: both are preparations which are applied on the skin (for external use) with the difference that topical products thought for a local action on one or more layers of the skin, and even if a part of the drug is able to reach the blood circulation, its concentration is sub-therapeutic, while, a transdermal formulation uses the skin to arrive at a systemic circulation [33].

Since 1979, when the Food and Drug Administration approved the first transdermal drug delivery system (Transderm Scop® Patch), up to the current transdermal delivery systems, a successful alternative to systemic drug delivery evolved. Despite their relatively higher costs, transdermal delivery systems have proved to be favored for the delivery of selected drugs, such as estrogens, testosterone, clonidine, nitroglycerin, scopolamine, fentanyl, and nicotine.

Compared to oral dosage forms, these systems offer not only an improved patient compliance, but also a superior uniformity of the drug concentrations in plasma throughout their duration of use. Most transdermal patches are designed to release the active ingredient at a zero-order rate for a period of several hours or days following the application to the skin [34].

1.6 Skin and cosmetics

To permit an active cosmetic ingredient to reach its target, it is essential that it is soluble in the type of tissue where it will reside [35]. Cells are composed by a lipophilic membrane but their interior is largely made of water and that is why a substance must be soluble in water to be able to stay there. On the other hand, when a cosmetic wants to reach the interior of a

cell, it must cross the lipid membrane. These aspects make cosmetic delivery rather complex. The cosmetic product must therefore have the right amount of oily substances and aqueous substances to cross cell membranes. The complexity of the design of a cosmetic active molecule is even greater because only certain chemical structures, including those of oily substances, can pass through the cells and other skin structures. For all these reasons, it is often necessary to include a substance that promotes intracellular penetration in cosmetic formulations [36]. The listed strategies can be useful for pharmaceutical and cosmetic topical formulations, but even more for the latter, in which the absorption problem is amplified by the low concentrations of the active ingredient used [37].

Strategies to improve the absorption of drugs and active ingredients in topical formulations are different:

Prodrugs: semi-synthesis reactions with the aim of increasing lipophilicity of molecules with an unfavorable partition coefficient to be absorbed by the stratum corneum [38].
Esterification reactions are often used. Prodrugs, after reaching the epidermis, are attacked by esterase enzymes and return water-soluble.

Supersaturated solutions: the biggest rate of skin penetration is achieved when a drug is at its highest thermodynamic activity as in a supersaturated solution. Equation 3 shows how this is possible in terms of thermodynamic activity [39]:

$$\frac{dm}{dt} = \frac{\alpha D}{yh}$$

Equation 3. *Skin penetatrion.*

Where α is the thermodynamic activity of the permeability in its vehicle and γ is the effective coefficient of activity in the membrane.
Supersaturated solutions may occur due to evaporation of solvent or by mixing of co-solvents. Topical formulations take advantage of this: when the formulation is applied on the skin, friction and the physiological temperature of the skin produce the evaporation of the solvent and the absorption of active ingredient. In addition, the aqueous component of the formulation works as anti-solvent (being absorbed by the skin) and the thermodynamic activity increases [40].

The main problems of this system are instability, risk of accumulation of the drug in the organism or in the skin and an increased cost of the formula.

Complexes: In the last twenty years, Drug-delivery complex structure is the most used strategy. In particular, the complexation of a drug with cyclodextrins. From the chemical point of view, there are different types of cyclodextrins but those most commonly used in the pharmaceutical field contain 6, 7 or 8 dextrose molecules (α, β or γ-cyclodextrin) linked together in a 1,4-configuration to form rings of different diameters.

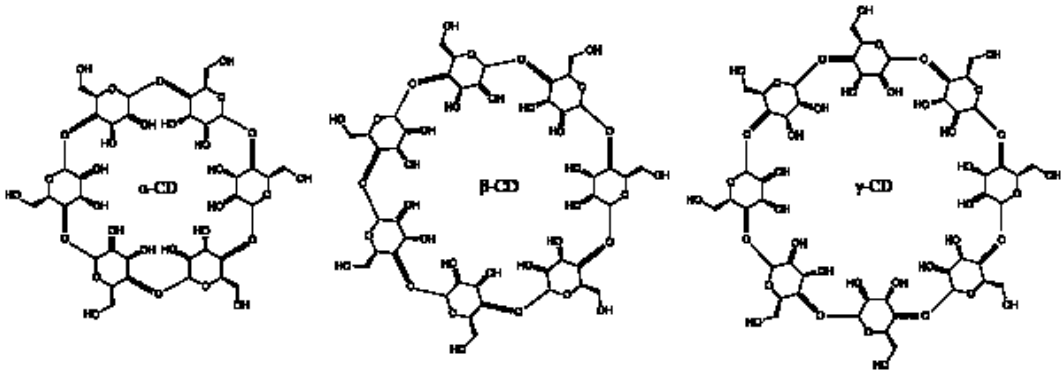


Figure 4. *α,β or γ-cyclodextrin.*

The ring has a lipophilic core and a hydrophilic external part in which organic molecules (with appropriate size) can form not-covalent inclusion complexes, with a consequent increase of solubility in water and a chemical stability[41].

The problem of cyclodextrins is that they are in equilibrium with the drug, therefore, in aqueous solutions they tend to release it; this is an advantage at the target situ but limits its use in hydrophilic formulations [42]. A good alternative to solve this problem is to form a covalent bonding between drug and cyclodextrin [43].

There are many examples of cosmetic products where the active ingredients are encapsulated in vesicles. These include humectants such as glycerol and urea, sunscreens and tanning agents, enzymes and also unstable active ingredients such as polyphenols [44].Currently, research is being directed towards the stabilization and reduction of the toxicity of molecules.

In this context, a strong impulse is given by some polymeric materials of natural, semi-synthetic or synthetic origin. Such polymers may be used as carriers: their function is to act as controlled release systems, to drop the active ingredients on the skin and to save it from degradation; polymers of this type can be biologically inactive or with intrinsic therapeutic

activity (to act directly as active ingredients, an example is cross-linked hyaluronic acid) [45].

Polymers as drugs, as carriers or excipients, allow us to obtain the following advantages:

- Therapeutic activity attenuated or delayed: This effect is very useful for high-potential therapeutic drugs, but highly toxic. The attenuation of the therapeutic effect is accompanied by a significant reduction in toxic effects. In some cases the impairment of ~~the~~ side effects can make a potentially toxic drug tolerable.

- Targeted Administration: instead of delivering an active ingredient to the bloodstream, which will move not only to the site of action, but also in the other parts of the body, the transport systems settle the drug directly on the target. This effect is useful in cases where the administered drug is harmful if present in high concentrations in the blood stream for possible actions in different compartments from those in which the therapeutic action is requested.

- Extended Activity: this effect is useful for unstable drugs in body fluids. The use of extended release systems of a drug allows to reduce the number of administrations and to stabilize the concentration of the drug in the blood.

- Synergistic effects of the activity of the drug connected with the presence of other drugs within the same macromolecular matrix: the possibility, therefore, to co-administer two different active molecules.

- Reduction of toxicity and side effects of conventional drugs: a sustained release over time consents to use a lower concentration of the drug, and therefore the risk of accumulation and side effects become less.

The use of polymers can, however, creates problems related to the following points:

- Destination of polymeric materials in the body: the use of a not biodegradable matrix often implicates an accumulation of high molecular weight

species in cellular lysosomes, leading to a lowered renal excretion of the macromolecular species.

- A polymer can give an immunological response by itself or by its metabolites. This is a tendency of natural or synthetic polymers to act as antigens, resulting in an induction of immune responses. The antigenic potential is closely related to their primary and secondary structure, in particular, a regular structure has a better chance of escaping the body's defense system.

Polymers are good delivery systems because they have the capacity of delivering the drug directly to the action site while minimizing collateral effects [46].

Principal structure investigates are: liposomes, nanoparticles (NP) and microparticles.

In order to ensure the controlled release of active ingredients, liposomes have gradually replaced conventional forms of release thanks to the following advantages: ability to protect the drug from degradation, drug ability to be directed to the site of action and reduction of collateral effects [47].

However, the production of liposomes has been limited because the encapsulation capacity is low. Furthermore, biodegradable NP have some specific advantages compared to liposomes, for example, they are able to increase the stability of drugs and proteins, and possess properties useful for the controlled release of active ingredients. NP generally have a range of dimensions between 10 and 1000 nm, the drug is dissolved, entrapped, encapsulated or attached to a polymeric matrix and depending on the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Nanocapsules are vesicular systems in which the drug is contained in a cavity surrounded by a single polymeric membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed.

Microspheres (also called microparticles) are small spherical particles of a diameter that goes from 1 to 1000 μm. Solid and hollow microspheres have very different density and therefore used for various applications: the hollow microspheres are typically used as additives to lower the density of a material, while the solid ones have numerous applications depending on the material of which they are made.

Microspheres of polystyrene and polyethylene are the two most common types of polymeric microspheres.

Polyethylene microspheres are characterized by a low melting point, and the availability of colored and fluorescent microspheres also make them desirable in the display and analysis of biological liquid flows in the various microscopy techniques. On the other hand, polystyrene microspheres are typically used in biomedical applications due to their ability to facilitate the procedures at a cellular level [48].

These systems widely vary in quality, roughness, uniformity of particle size distribution, and, for this reason, each application requires appropriate microspheres.

The range of techniques for the preparation of microspheres offers different opportunities to control various aspects of the administration of a drug. This approach facilitates the accurate delivery of small quantities of potent drugs, reduces the concentration of the drug in a different site than the predetermined one and also protects the drug before and after administration.

Other common compositions include microspheres of polystyrene (PS), polymethylmethacrylate (**PMMA**) and silica. These materials possess different physical and optical properties which in turn represent advantages or limitations in the application field.

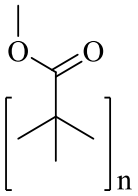
Polymer particles are generally hydrophobic so they have a high binding capacity to proteins. Nevertheless, they often require the use of certain surfactants such as 0.01- 0.1% Tween 20 or SDS in the storage buffer in order to provide greater manageability [48].

Silica aqueous solutions rarely require the use of surfactants or other stabilizers.

Conventionally, these systems can be prepared using two methods: the loading of the active ingredient within a preformed polymer and polymerization in situ, the construction of the polymer from its monomers around the active substance itself.

Several are the pharmaceutical applications of these systems in drug delivery: oral administration, ophthalmic, nasal, buccal, but also as topical application at a vaginal and gastrointestinal apparatus level[48]. In cosmetics, different range of polymers are applied as film formers, fixatives, rheology modifiers, associative thickeners, emulsifiers, stimuli-responsive agents, conditioners, foam stabilizers and destabilizers, skin-feel beneficial agents, and antimicrobials[49].

1.7 Polymethylmethacrylate (PMMA)



Polymethylmethacrylate (**PMMA**) is a plastic material made from polymers of methyl methacrylate, ester of methacrylic acid.

It is a not a biodegradable polymer but biocompatible [50]; and because of its lipophilic nature, it preferably includes hydrophobic drugs, thereby guaranteeing better skin penetration.

PMMA has a high degree of compatibility with human tissue and, for this reason, it is widely used in the field of application. It is well known, for example, in the production of intraocular lenses for the treatment of cataract and in the production of rigid contact lenses [51].

PMMA is also successfully used in the field of dermatology, where recent studies suggest the beneficial action of **PMMA** microspheres for the treatment of atrophic acne scars [52]. Even in cosmetics, **PMMA** is already used to obtain the mat effect in makeup, exploiting its ability to absorb oily and fatty secretions.

2. AIM OF THE WORK

The use of topical formulations is increasing, not only for cosmetic application, but also in pharmaceutical field because it allows a targeted and localized application.

The production of a topical formulation is complicated by several aspects; stability and release of the active ingredient are crucial points. Another critical point is the passage across the skin that works as a protective barrier for our body and last but not least the compliance (skin feeling). It is however possible to modulate the release and consequent absorption of an active from a topical formulation playing on different factors such are modification of chemico-physical properties of the molecule, changing excipients or using stabilizing structures with inclusion of the active.

The present PhD project is addressed to the investigation of different aspect of topical formulations. It mainly consists in two parts, dealing with new strategies for the preparation of novel ingredients for topical application, the study of the release, stability and activity of active ingredients (pharmaceutical or cosmetic ones) in topical formulations. In particular the project was dedicated in the first part to the preparation and evaluation of new molecules for topical applications, in the second part, conducted during a one-year period spended at the R&D facilities of Angelini Farmaceutici Spa in Ancona, dealing with the study of the topical delivery of pharmaceutical and cosmetic molecule.

Four main studies were conducted:

1. Synthesis and evaluation of biological activity of molecules with dualistic properties, characterized by the benzymidazolic ring, in order to obtain, at the same time, compounds with UV-filtering and antioxidant activity. This would allow to protect us from the short and long-term damage to the skin of the solar radiation.

2. Preparation of semi-synthetic derivatives of Hesperidin, in order to obtain more lipophilic molecules. Synthesized molecules and natural ones were then tested and compared in terms of biological activity and stability in cosmetic formulations.

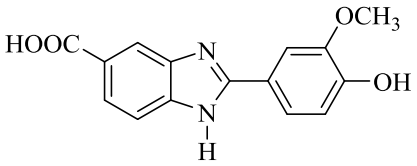
3. Baicalin and Salicin, two substances with a high therapeutic potential but not very stable and difficult to use in topical application; in order to improve the absorption of these compounds in biological membranes and to facilitate their incorporation in topical formulations, we followed two different strategies. The first one was the protection of OH groups, through esterification reactions, to obtain pro-drugs. The second strategy had been to incorporate the active ingredients into polymeric structures and then study their release.

4. Use of polimers to increase topical stability and delivery. Polymethylmetacrilate as delivery system: we studied loading method and polymer's performances in topical formulation. The goal was a modified release of the molecule by molecule-polymer complex and/or an increase of the stability of the drug in the formulation. Complexes tested and formulations were both cosmetic and pharmaceutical.

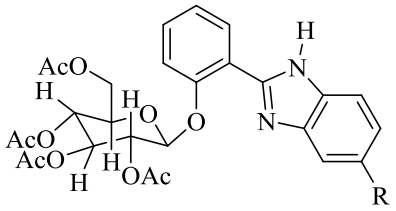
3. RESULTS and DISCUSSION

3.1 Benzymidazolic compounds

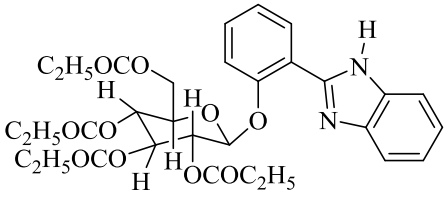
With the intent to obtain compounds with dualistic topical activity, as sunscreen and antioxidant, we synthetized four compounds, all with benzymidazolic ring, one bearing free phenolic functionality (**AB101**) and three with phenol group protected by a glycosidic bond (**AB102-104**).



AB101



R=H **AB102**
R=COOH **AB103**



AB104

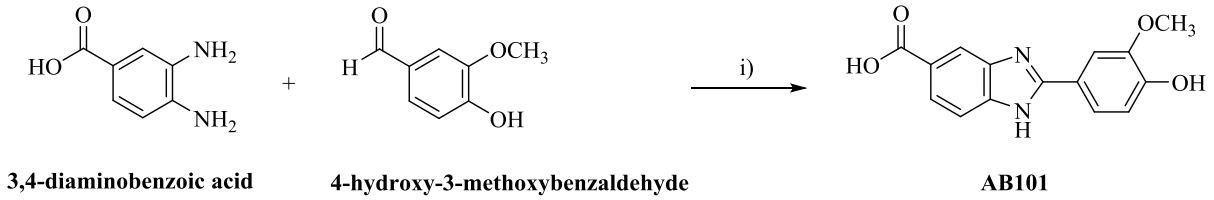
Scheme 1. *Synthetic compounds with Benzymidazolic ring.*

3.1.1 Synthesis of 2- (4-hydroxy-3-methoxyphenyl) -1H-benzo [d] imidazole-5-carboxylic acid (AB101)

Synthesis of this derivative involved the use of 3,4-diaminobenzoic acid with 4-hydroxy-3-methoxybenzaldehyde.

Various procedures described in literature had been evaluated, in order to find an cheap way to obtain the desired product with good yield. The method chosen involved the use of sodium bisulfite ($NaHSO_3$) in Ethanol (EtOH) at reflux for 24h [53]. **AB101** was obtained according to this procedure in 17h, but using Methanol (MeOH) in place of the original

solvent (Scheme 2). In this way the product was obtained in less time (probably for the higher acidity of MeOH), but also with a solvent economically more accessible.

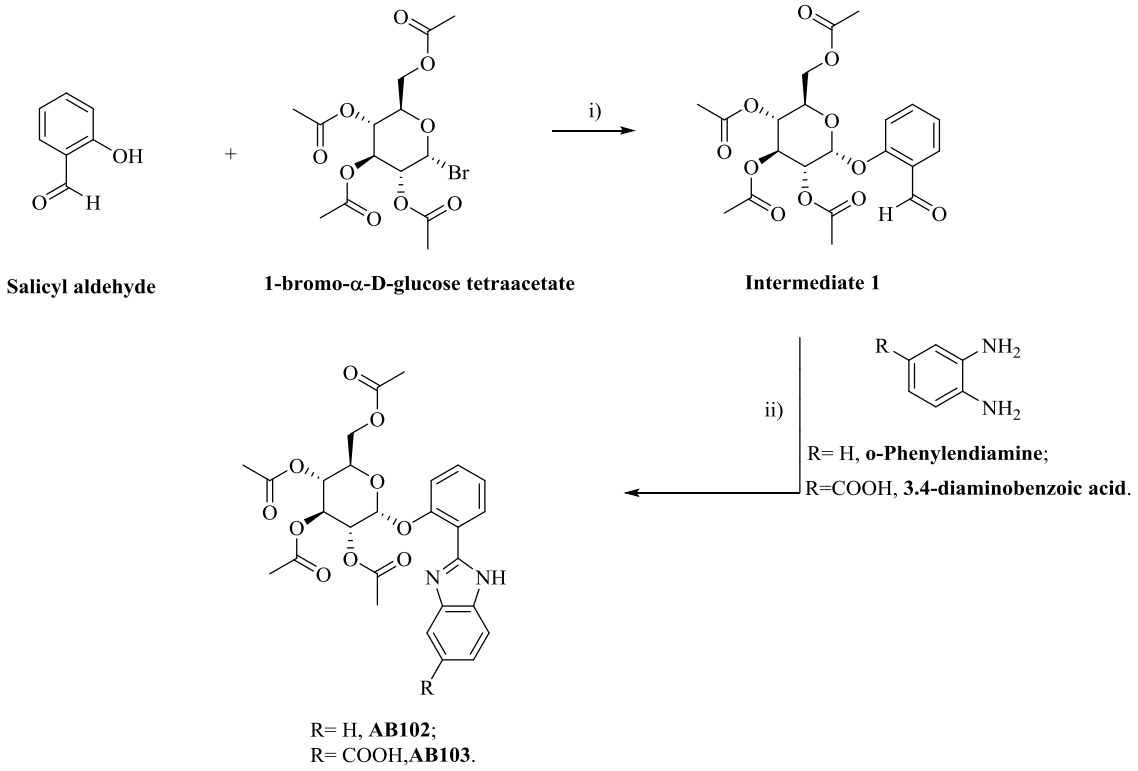


3,4-diaminobenzoic acid **4-hydroxy-3-methoxybenzaldehyde** **AB101**

Scheme 2. *Synthesis of* **AB101**. *i) $NaHSO_3$, 80°C, MeOH (17 h).*

3.1.2 Synthesis of 2- (2-hydroxyphenyl-β-o-D-glucose tetraacetate) -1H-benzo [d] imidazole (AB102) and 2- (2-hydroxyphenyl-o-β-D-glucose tetraacetate) -1H-benzo [d] imidazole-5-carboxylic acid (AB103).

In order to obtain compounds similar to the product **AB101** in which the phenol was protected by glycosidic bond, salicyl aldehyde (or 2-hydroxybenzaldehyde) was reacted with 1-bromo-α-D-glucose tetraacetate, according to the conditions shown in Scheme 3. This synthetic procedure for the glycosylation [54] comprised a solid-liquid two-phase system consisting of potassium carbonate (solid, K_2CO_3) and chloroform (solvent, $CHCl_3$) in the presence of an ammonium salt (chloride benzyltributylammonium, $C_6H_5CH_2(C_4H_9)_3NCl$) as phase transfer catalyst maintained under stirring for 24h at room temperature. In place of the chloride benzyltributylammonium, benzyltriethylammonium chloride ($C_6H_5CH_2(C_2H_5)$ NCl) [55] was used, in anhydrous environment.



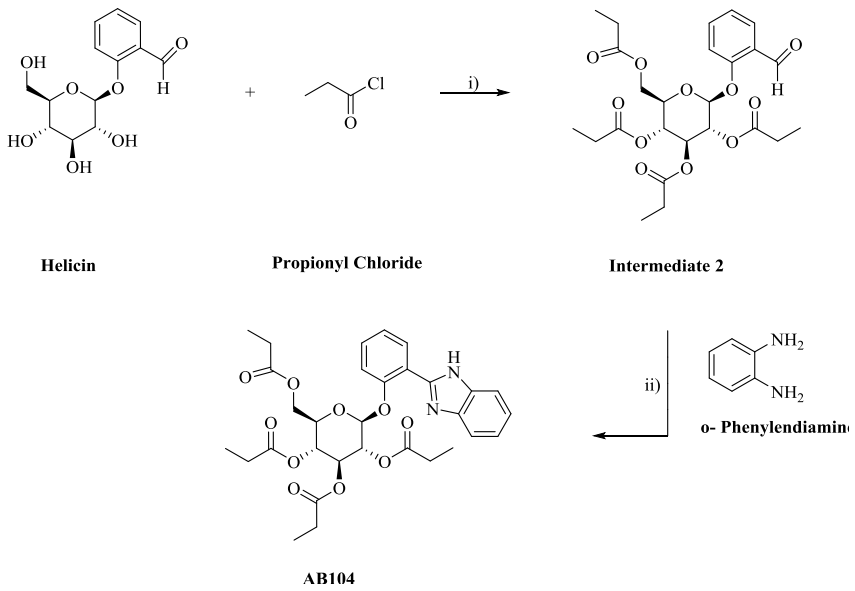
Scheme 3. *Synthetic procedure of* **AB102**, **AB103. i) $C_6H_5CH_2(C_2H_5)NCl$, K_2CO_3, anhydrous CH_2Cl_2 , (24 h); ii) $NaHSO_3$, 80°C, MeOH (R=H: 3h;R=COOH: 2h).**

In the successive cyclization (ii) the intermediate **1** was reacted with either o-phenylenediamine, with 3,4-diaminobenzoic acid to form benzymidazole ring, respectively obtaining the products **AB102** and **AB103**. The presence of the carboxylic acid substituent on **AB103** should provide a bathochromic effect as to shift towards the absorption range of UVA.

3.1.3 Synthesis of 2- (2-hydroxy-phenyl-o-β-D-glucose tetra propionate) - 1H-benzo [d] imidazole (AB104)

An alternative procedure to obtain analogues of compounds **AB102** and **AB103** was adopted. For this purpose, glycosilated salicylaldehyde (salicilaldehyde β-D-glucoside, known as Helicin, was used as starting material whose hydroxyl groups have been previously esterified using an acyl chloride in presence of 4-dimethylaminopyridine (4-

DMAP), in an inert atmosphere, to obtain the derivative tetrapropionate (Intermediate **2**) [56].



Scheme 4. *Synthetic procedure of* **AB104. i) 4- DMAP, anhydrous CH_2Cl_2 , (24 h); ii) $NaHSO_3$, 80°C, MeOH (2h).**

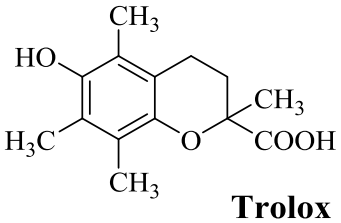
The purified intermediate was then reacted with o-phenylenediamine according to the procedure already described for the synthesis of the product **AB102**.

3.1.4 Antioxidant Activity

PCL method (photochemiluminescence) is based on the amplification of radicals of reaction resulting from reactive oxygen species (ROS) caused by photoinduced oxidation of Luminol, which is accompanied by an intense chemiluminescence. The presence of antioxidants inhibit the autoxidation of luminol dues to the interaction with the superoxide anion ($O_2^{\cdot -}$), generated by photochemical route. This test [57] is very quickly (3 minutes) and sensitive (order of nanomoles) and can be carried out according two different protocols: ACW (Antioxidant Capacity Watersoluble) and ACL (Antioxidant Capacity Lipidsoluble), allowing to quantify, for the same product complex, either the antioxidant

capacity of water-soluble component (flavonoids, vitamin C, etc.) and of the lipophilic ones (Tocopherols, tocotrienols, carotenoids, essential oils etc.) [58].

PCL method measures antioxidant capacity against $O_2^{\cdot-}$, one of the most dangerous reactive oxygen species responsible for oxidative damage to biological structures directly related to adverse effects on human health. PCL also allows to evaluate the antioxidant capacity of complex mixtures or pure substances by the construction of a calibration curve with a reference antioxidant. In our case we used as reference, for the protocol in a lipophilic environment (ACL), Trolox®, a potent analogue of vitamin E, expressing the powers obtained as micromoles equivalent in antioxidant activity of Trolox®.



Trolox

PCL protocol in an aqueous environment (ACW) uses Ascorbic acid as reference, antioxidant activity is expressed as micromoles equivalent in antioxidant activity of Ascorbic Acid on gram of tested product.

The determinations were conducted according to the method originally described by Popov and Lewin [59].

AB101, **AB102**, **AB103** e **AB104** were tested to determine their antioxidant capacity by PCL analysis.

Compounds	PCL µmolTE/g
AB101	1367.521 ± 9.8
AB102	5.7681 ± 0.4
AB103	6.027 ± 0.5
AB104	4.816 ± 0.6

Table 1. *PCL test on **AB101**, **AB102**, **AB103** and **AB104**. Each value was obtained from three different experiments (Mean ± SE).*

AB101, the only one with a free phenolic functionality, had a good antioxidant activity, while compounds **AB102**, **AB103** and **AB104**, in which all the phenolic functionality were protected, had low antioxidant activity. At this point it will be interesting, especially for the last three compounds, the possibility of an *in vivo* test for antioxidant activity: the presence in the organism of enzymes able to split glycosidic bond, could break phenolic functionality, allowing to observe antioxidant capacity.

3.1.5 Antifungal Activity

Dermatophytes [60] are pathogenic fungi that have a particular affinity for keratin and thus attacking skin, hair and nails, on which cause infections named dermatophytosis (ringworm) due to digestion of keratin (keratolytic activities).
The main forms of ringworm [61] are: *Tinea pedis* (desquamation and maceration of feet), *Tinea unguium* (abnormalities of color and texture of the nail plate), *Tinea cruris* (inguinal and genital infection characterized by itching, redness and flaking), *Tinea capitis* (scalp infection), and *Tinea corporis* (infection of the skin accompanied by inflammatory reactions). The dermatophyte fungi used in the analysis are:

- *Trichophyton rubrum*: cosmopolitan fungus, but mostly prevalent in city environments; it is responsible for *Tinea unguium*, *Tinea pedis* and *Tinea cruris*, and it can also result in chronic infections;

- *Trichophyton mentagrophytes*: It is found throughout the world and it is both anthropophilic and zoofila species, which causes inflammation of the hair follicles.

- *Arthroderma cajetani*: causes infections on dogs and human.

- *Microsporum canis*: usually infects animals, especially dogs and cats, spreading infectious particles in the domestic environment, determining potential infections to the family components, especially children, on which manifested ringworm of the scalp.

- *Microsporum gypseum*: causes ringworm in the body, nails, and scalp, and it is a popular fungi in the world; the main source of infection for humans and animals is the ground, and in fact it causes infection of gardeners and farmers.

- *Trichophyton tonsurans*: predominantly it affects men and does not determine inflammatory reactions.

- *Epidermophyton floccosum*: It has spread throughout the world and it is one of the main responsible for tinea pedis and tinea cruris, which affects feet and inguinal areas, especially in men, but also in women; it is certain that this fungus does not affects hair.

- *Trichophyton violaceum*: mainly it affects children and causes tigne of the scalp, but it can also develop into epidermal.

AB101, **AB102**, **AB103** and **AB104** had been studied for their antifungal activities against eight fungal species causing mycosis.

		% Inhibition of growth			
Fungus		**AB101**	**AB102**	**AB103**	**AB104**
Epidermophyton floccosum	20 µg/ml	11.3 ± 0.9	3.5 ± 0.2	0.00	4.5 ± 0.3
	100 µg/ml	19.4 ± 1.7	10.3 ± 0.6	5.1 ± 0.2	7.5 ± 0.7
Trychopyton rubrum	20 µg/ml	13.6 ± 1.1	+	0.8 ± 0.07	11.8 ± 1.1
	100 µg/ml	15.3 ± 0.7	+	4.9 ± 0.3	17.7 ± 1.0
Trichophyton tonsurans	20 µg/ml	+	8.8 ± 1.3	+	1.7 ± 0.09
	100 µg/ml	12.1 ± 1.6	11.8 ± 0.8	+	13.3 ± 1.2
Tricophyton violaceum	20 µg/ml	+	10.7 ± 0.9	2.1 ± 0.1	10.0 ± 0.5
	100 µg/ml	+	15.8 ± 1.2	7.3 ± 0.5	22.5 ± 1.6
Tricophyton mentagrophytes	20 µg/ml	+	+	+	14.4 ± 1.2
	100 µg/ml	+	24.5 ± 1.9	5.6 ± 0.4	15.3 ± 1.3
Mycrosporum gypseum	20 µg/ml	+	+	24.3 ± 1.2	15.8 ± 1.4
	100 µg/ml	+	+	32.1 ± 1.3	22.8 ± 1.9

Arthroderma cajietani	20 mg/ml	+	7.8 ± 0.6	+	15.2 ± 1.4
	100 µg/ml	+	15.5 ± 1.1	+	19.6 ± 1.7
Mycrosporum canis	20 µg/ml	+	0.7 ± 0.001	7.8 ± 0.6	27.6 ± 1.6
	100 µg/ml	+	24.7 ± 1.8	8.6 ± 0.4	28.9 ± 1.9

Table 2. *Percent growth inhibition of dermatophytes treated with **AB101**, **AB102**, **AB103** and **AB104** at 20 or 100 µg/mL. Each value is the mean of three measurements.*

As showed in Table 2, antifungal activity of **AB101**, **AB102**, **AB103** e **AB104** were not interesting: in all cases the percentages of growth inhibition were rather low and the highest value was 32.1% of inhibition reached by **AB103** on *Microsporum Gypseum* at the highest dose. In other cases all the substances showed an hormone-like effect with a fungal growth higher than that of the controls (+).

3.1.6 Photoprotective activity

The photoprotective activity of compounds **AB101**, **AB102**, **AB103** and **AB104** in co-formulations with avobenzone (3%) and octyl methoxycinnamate (6%) were determined in terms of SPF, UVAPF and Critical Wavelength (λ_c), so as to determine complete UV spectrum of protection.

Critical Wavelength is a parameter that determines if a filter have a broad spectrum of protection, is defined as the shortest wavelength in which the absorption product corresponds to 90% of the total absorption. Critical Wavelength also describes the UVA protection properties of a product, to be indicated as appropriate in protecting against UVA radiation, should have a value of λc greater than 370nm.

SPF value was obtained from the ratio between the time of exposure to UVB radiation necessary to develop a site erythema on skin protected by the sunscreen and the time required to produce the same effect on a site bare skin, without the application of any product [62]. UVAPF refers to protection against UVA radiation, which is important as UVA rays penetrate into the deeper layers of the skin and damage DNA and tissues through the production of reactive oxygen species (ROS) [63].

In this work, the photo protective activity of the conjugates was determined in terms of SPF, UVAPF and Critical Wavelength, with the objective to determine the full UV spectrum protection. To have a first estimate of Critical Wavelength, we had prepared and analyzed EtOH solutions of our compounds to 3% by weight. Spectra were recorded at 290-400 nm and the λ_c was calculated. Results were shown in Table 3.

Compounds	λ_c(nm)
AB101	340
AB102	324
AB103	328
AB104	323

Table 3. *λ_c values of the conjugates **AB101-104**.*

None of these molecules could be considered a broad-spectrum filter, because the critical Wavelengths were below 370 nm.

So each of four compounds synthesized were inserted in basic formulations containing a combination of two filters, to evaluate the possibility of a synergistic action.

We prepared four formulations based on 3% avobenzone (AB) and 6% in octyl methoxycinnamate (OMC) and each of them had been enriched with synthetic compounds at a concentration of 1%. The formulation which contained only AB and OMC had been used as blank.

Absorption spectra for each formulation and the data obtained were tabulated in Table 4.

Formulation	SPF	UVAPF0
A. 3% AVB + 6% OMC	13,52	7,26
B. 3% AVB + 6% OMC + 1% **AB101**	12,44	6,85
C. 3% AVB + 6% OMC + 1% **AB102**	14,02	7,45
D. 3% AVB + 6% OMC + 1% **AB103**	18,6	8,96
E. 3% AVB + 6% OMC + 1% **AB104**	13,64	7,26

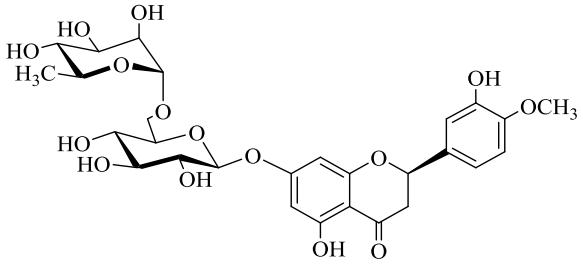
Table 4. *SPF values of formulations with synthetic compounds.*

From the data presented, formulations with **AB101** and **AB104** did not show any synergistic effect: compared to the reference formulation, critical Wavelength and SPF values were not significantly varied. **AB102** had a negative effect, with the SPF lowering of a unit. On the contrary compound **AB103** had a very significant increase of 5 units in the SPF value.

This result showed that the presence of a carboxyl group (COOH) give to the molecule a bathocromic effect.

The very low percentage with which was present in the formulation indicates a good potential for the molecule at issue.

3.2 Hesperidin derivatives



Flavonoids are an important group of phenolic compounds that are widely distributed in plants. In literature, a lot of flavonoids has been tested both in their free form and in the glycosylated form.

Hesperidin is a flavanone, constituted by Hesperetin, to which it is linked in position 7 the rutinose sugar, a disaccharide consisting of glucose and rhamnose (7-O-beta-D-ramnoglucose). It is extracted in large quantities from the peel and seeds of fruits of *Citrus aurantium*, *Citrus limon*, *Citrus reticulata*, *Citrus sinensi*, *Citrus tangerina*, *Citrus tangelo*.

The concentration of Hesperidin in the fruit grows gradually to the ripening stages of the same [64].

Hesperidin is present abundantly in citrus fruits and for its numerous biological properties is also called bioflavonoid [65]. The main features that are recognized to Hesperidin are a vitamin-like activity and the ability to reduce the permeability, weakness and capillary

fragility (vitamin P). It also shows antioxidant properties, anti-inflammatory, anti-cancer and anti-allergic [66]. Hesperidin, as generally happens for the polyphenols, is a molecule with numerous physiological and pharmacological activities but its use is limited by poor stability, difficulty in formulation as well as skin absorption because of its extremely low lipophilicity.

In order to extend its potential applications, we synthesized seven esterified derivatives (Table 3) with groups of different nature to explore lipophilicity changes. Each of the derivatives obtained had none, one or more free OH phenolic group in order to evaluate the differences, in terms of biological activity and stability.

Esterification reactions were carried out using the different groups in the presence of 4-dimethylaminopyridine (4-DMAP) in CH_2Cl_2. All derivatives were obtained by the same synthetic procedure (Table 5) but they were purified with different methods.

Compounds	R	R1	R2
Esp1	$-C_6H_{11}O$	-OH	$-C_6H_{11}O$
Esp2	$-C_8H_{13}O$	-OH	$-C_8H_{13}O$
Esp3	$-C_7H_5O$	-OH	$-C_7H_5O$
Esp4	$-C_7H_{13}O$	-OH	$-C_7H_{13}O$
Esp5	$-C_3H_5O$	-OH	$-C_3H_5O$
Esp6	$-C_{11}H_7O$	-OH	$-C_{11}H_7O$
Esp7	$-C_9H_7O$	$-C_9H_7O$	$-C_9H_7O$

Table 5. *Synthetic derivatives of Hesperidin.*

3.2.1 Partition Coefficient Octanol / Water

The determination of the partition ratio (LogP), was calculated as the logarithm of the concentration of substance that was in octanol and water. The test was conducted in a biphasic system between octanol (20mL) and water (20mL pH 12) at 25 ° C. After 30 minutes of stirring, the biphasic mixture was centrifuged for 10 minutes at 4000 rpm to separate the phases and then about 5 ml of each phase was used for the determination with spectrophotometer (λmax fixed for each single compound).

Compounds	*LogP**
Hesperidin	-0.87
Esp1	1.38
Esp2	0.40
Esp3	2.04
Esp4	1.18
Esp5	1.08
Esp6	1.08
Esp7	1.04

* LogP = log10 [mg/20 mL]octanol/[mg/20 mL]H_2O

Table 6. *LogP values of Hesperidin and its synthetic derivatives.*

Results obtained (Table 6) confirmed the increased lipophilicity of synthetic derivatives than Hesperidin that had a negative value of LogP.

3.2.2 Antifungal activity

Hesperidin and its derivatives were tested as antifungal activity on six dermatophytes by the diffusion method in Sabouraud Dextrose Agar (SDA), using DMSO as a solvent. (*see paragraph 3.1.5*).

		% Inhibition of growth			
Fungus		**Hesperidin**	**Esp1**	**Esp4**	**Esp5**
Epidermophyton floccosum	20 µg/ml	+	3.3 ± 0.2	+	2.5 ± 0.2
	100 µg/ml	+	10.0 ± 0.4	1.3 ± 0.2	5.0 ± 0.4
Trichophyton tonsurans	20 µg/ml	1.4 ± 0.5	+	+	0.00
	100 µg/ml	4.2 ± 0.8	5.6 ± 0.3	2.7 ± 0.3	+
Tricophyton mentagrophytes	20 µg/ml	0.00	+	+	+
	100 µg/ml	9.8 ± 0.8	+	+	+
Mycrosporum gypseum	20 µg/ml	+	0.8 ± 0.07	0.00	+

	100 µg/ml	5.4 ± 0.5	3.2 ± 0.2	0.7 ± 0.03	2.2 ± 0.5
Arthroderma cajietani	20 mg/ml	+	+	0.8 ± 0.01	+
	100 µg/ml	+	8.5 ± 0.6	2.4 ± 0.5	+
Mycrosporum canis	20 µg/ml	0.00	+	+	5.9 ± 0.6
	100 µg/ml	1.9 ± 0.4	+	+	9.9 ± 0.8

Table 7. *Percent growth inhibition of dermatophytes treated with **Hesperidin**, **Esp1**, **Esp4**, and **Esp5** at 20 or 100 µg/mL. Each value is the mean of three measurements.*

		% Inhibition of growth			
Fungus		**Esp2**	**Esp3**	**Esp6**	**Esp7**
Epidermophyton floccosum	20 µg/ml	1.8 ± 0.02	7.7 ± 0.6	+	0.00
	100 µg/ml	7.1 ± 0.8	15.4 ± 0.9	0.00	2.9 ± 0.3
Trichophyton tonsurans	20 µg/ml	4.0 ± 0.8	+	+	+
	100 µg/ml	12.0 ± 1.1	+	3.2 ± 0.2	+
Tricophyton mentagrophytes	20 µg/ml	0.00	+	+	0.00
	100 µg/ml	2.8 ± 0.1	+	+	3.6 ± 0.5
Mycrosporum gypseum	20 µg/ml	0.00	+	+	4.9 ± 0.5
	100 µg/ml	4.1 ± 0.3	+	+	8.6 ± 0.9
Arthroderma cajietani	20 mg/ml	+	+	+	2.9 ± 0.2
	100 µg/ml	+	4.7 ± 0.6	0.8 ± 0.05	7.3 ± 0.6
Mycrosporum canis	20 µg/ml	6.0 ± 0.3	0.9 ± 0.06	+	2.1 ± 0.3
	100 µg/ml	16.0 ± 0.9	3.7 ± 0.4	1.6 ± 0.2	5.0 ± 0.5

Table 8. *Percent growth inhibition of dermatophytes treated with **Esp2**, **Esp3**, **Esp6**, and **Esp7** at 20 or 100 µg/mL. Each value is the mean of three measurements.*

Antifungal activity of the natural molecule and its derivatives (Table 7, Table 8) did not result to be very interesting: in fact, in all cases the percentage inhibition values were less than 20%. In particular Hesperidin showed lowest activity on dermatophytes compared to synthetic compounds with the exception of the concentration 100 µg/mL on *Trichophyton mentagrophytes*.

3.2.3 Antioxidant Activity

To evaluate the antioxidant activity of semisynthetic compounds and Hesperidin, we used three different tests using three various radicals, so we could have a wider spectrum of action. Tests were DPPH, FRAP and PCL (*paragraph 3.1.4*).

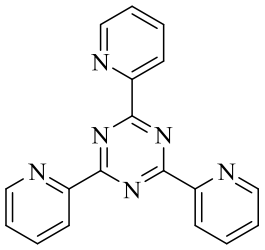
Compounds	PCL µmolTE/g
Hesperidin	1068.63 ± 1.12
Esp1	2.16 ± 0.09
Esp2	1.14 ± 0.50
Esp3	22.58 ± 2.92
Esp4	12.04 ± 2.20
Esp5	9.15 ± 0.82
Esp6	6.06 ± 1.70
Esp7	8.12 ± 1.83

Table 9. *PCL analysis.* Each value was obtained from three experiments (Mean ± SE).

Table 9 shows antioxidant activity of Hesperidin and **Esp1- Esp7** by PCL analysis.
Hesperidin had a elevated antioxidant activity (1068.63 µmolTE/g), but synthetic derivatives had very lower values.

3.2.3a Frap Test

FRAP test (Ferric Reducing Antioxidant Power) is suitable for testing the antioxidant capacity of both complex mixtures and pure substances. It is a simple and inexpensive test developed by Benzie and Strain to measure the plasma antioxidant power [67], which is based on the ability of antioxidants to reduce, in an acid environment, ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in the presence of TPTZ (2,4,6-tripyridyl-triazine) (Figure 4).



2,4,6-Tri(2-pyridyl)-s-triazine

Figure 4. *TPTZ radical.*

Frap test is based on this reaction:

$$Fe(TPTZ)_2(III) + ArOH \longrightarrow Fe(TPTZ)_2(II) + ArOH^{\bullet+}$$

In absence of antioxidant, the Fe^{3+}-TPTZ complex has a brown color. When the antioxidant solution is added to the acidic solution of ferric tripyridyl-triazine, this instantly assumes an intense blue coloration, due to the reduction of ferric ion to ferrous ion by antioxidant molecule, with formation of the complex Fe^{2+}-TPTZ, which shows an absorption maximum at 593 nm. A change in absorbance of the test compounds give a measure of their reducing ability [68]. The absorbance change is related to the total reducing power of antioxidant electron present in the reaction.

FRAP method allows to evaluate the antioxidant capacity using calibration curve with a reference antioxidant, Trolox.

Compounds	FRAP µmolTE/g
Hesperidin	1255.32 ± 4.04
Esp1	44.37 ± 0.96
Esp2	20.15 ± 0.58
Esp3	14.18 ± 0,64
Esp4	12.13 ± 1.12
Esp5	16.28 ± 0.46
Esp6	19.61 ± 0.84
Esp7	18.19 ± 0.77

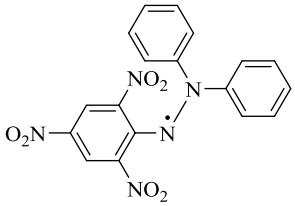
Table 10. *Evaluation of antioxidant capacity of Hesperidin and synthetic derivatives by FRAP test. Each value was obtained from three experiments (Mean ± SE).*

The analysis carried out by the FRAP test confirmed high antioxidant capacity of Hesperidin and instead much lower values for the semi-synthetic derivatives. In particular, as shown in Table 10, the derivatives that had bulky substituents such as alkyl groups (**Esp1** and **Esp2**) had demonstrated a greater capacity of inhibition against the radical TPTZ compared to aromatic derivatives.

3.2.3b DPPH Test

This test calculates the antioxidant activity of compounds against DPPH· radical (1,1-diphenyl-2-picrylhydrazyl radical) which is one of the most stable nitrogen radicals and chromophore.

The solutions prepared with this substance shows a purple color, but when the DPPH is reduced by a hydrogen donor substance, the solution changes color to assume the pale yellow coloring.



1,1-Diphenyl-2-picrylhydrazyl radical

Figure 5. *DPPH radical.*

This test is based on measuring the reducing activity of antioxidants against the radical DPPH (Figure 5).

The antioxidant power is then determined by measuring the decrease in absorbance of the DPPH radical at a wavelength of 517 nm, with a UV-visible spectrophotometer, after reaction with the test samples.

The percentage of DPPH radical remaining · is calculated as follows:

$$\%DPPH\cdot_{rim} = 100 \times [DPPH\cdot]_{rim} / [DPPH\cdot]_{T=0}$$

The percentage of remaining DPPH radical (DPPH $\cdot_{rim}$) is inversely proportional to the concentration and the antioxidant capacity of the test compound: the concentration of product which causes a decrease of the initial DPPH · of 50% is defined as IC50. The time needed to reach the IC50 is defined TIC50. The absorbance measurements of the samples examined were performed with a UV-VIS spectrophotometer, following the method described by Wang et al. [69].

Compounds	DPPH µmolTE/g
Hesperidin	216,61 ± 0,87
Esp1	No activity
Esp2	No activity
Esp3	6.03 ± 0.31
Esp4	2.86 ± 0.52
Esp5	2.93 ± 0.81
Esp6	No activity
Esp7	No activity

Tabella 11. *DPPH analysis of Hesperidin and its derivatives. Each value was obtained from three different experiments (Mean ± SE).*

As it can be seen from the values given in Table 11, none of synthetic derivatives displayied significant values in terms of antioxidant activity against DPPH radical; on the contrary, Hesperidin was found to be very powerful.

3.2.4 Stability studies

The synthetic derivatives of Hesperidin were mainly synthesized in order to be pro-drugs: to make its more stable in formulation and more bioavailable. For this reason it was decided to evaluate their stability within dermo-cosmetic formulations with 0.3% (w/w) of each molecules. As a preliminary analysis, we selected only five of the seven derivatives obtained.

Derivatives **Esp4** and **Esp5** have not been tested because the yield was very low, and there was enough time for optimization.

Formulations containing active ingredients, were subjected to accelerated aging at 40°C for 3 months and monitored by HPLC to evaluate the stability of Hesperidin and derivatives in function of time. The results obtained were reported in Figure 6.

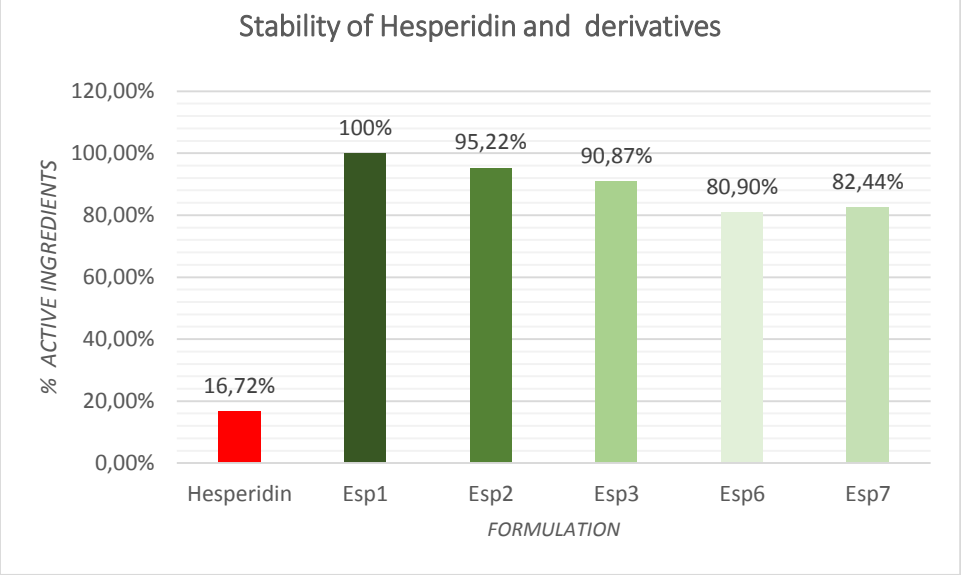


Figure 6. *T120 Stability data of formulations with Hesperidin and its derivatives (Results are percentage decrease compared to T0).*

Figure 6 shows that Hesperidin was unstable compared to all tested derivatives. In fact we observed a maximum percentage decline of 20% at the 120th day of analysis for the derivatives, while Hesperidin, by contrast, showed a decrease of 85%.

3.3 Baicalin, Salicin and their semisynthetic derivatives

3.3.1 Synthesis of Baicalin derivative (B1)

Baicalin is a flavonoid derived from *Scutellaria baicalensis* plant. It is a molecule with important pharmacological activities, such as antioxidant and anti-inflammatory activity, antiviral, antimicrobial, antitumor, besides the action of the molecule on the central nervous system [70].

Unfortunately also Baicalin, like Hesperidin, is poor stable in biological fluids [71] and in formulation, in particular to pH changes. Specifically, the main form of degradation concerns oxidation-reduction reactions at the level of phenol by radical species. For this reason we synthesized a derivative with all the hydroxyl groups protected through the use of acetyl groups. Because of the instability of Baicalin in different reaction conditions,

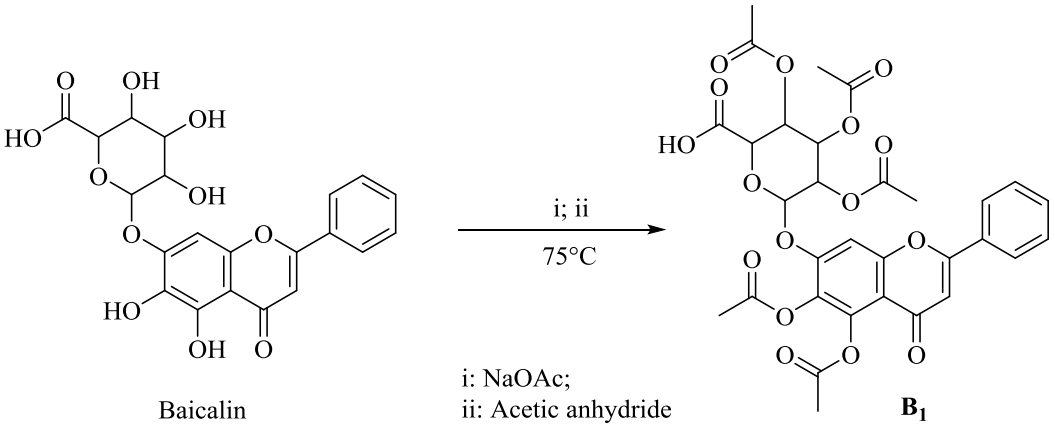
before being able to obtain the derivative of interest, we tried three different synthetic strategies:

1. Esterification reaction in anhydrous conditions with propionyl chloride and DMAP in methylene chloride;

2. Esterification reaction in anhydrous conditions with propionyl chloride and pyridine in methylene chloride;

3. Esterification reaction in anhydrous conditions, in presence of propionic acid in sulfuric acid.

The synthetic strategy that had allowed us to obtain the derivative **B1**, involved the use of sodium acetate in acetic anhydride at reflux (75 ° C) (Scheme 5).



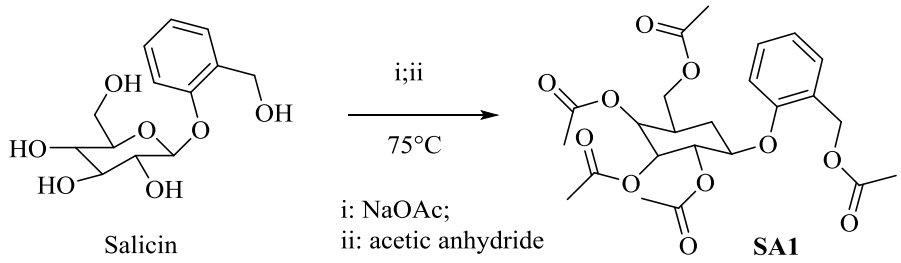
Scheme 5. *Acetylation of Baicalin.*

3.3.2 Synthesis of Salicin derivative

Salicin is a glucoside, made up of glucose and salicylic alcohol, mainly extracted from plants of *Salix alba* [72]. From pharmacological point of view, it belongs to the class of Salicylates and, as such, showed antipyretic activity [73], anti-inflammatory and analgesic

activity by inhibition of the COX [74]. Salicin is a highly hydrosoluble compound and so it is poorly used in anhydrous media.

All of Salicin hydroxyl functionalities had been esterified using sodium acetate in Acetic Anhydride to obtain the semi-synthetic derivative **SA1** (Scheme 6). This modification allows to obtain a derivative that, while maintaining the pharmacological quality of the starting product, performs like a pro-drug, improving the stability of the active ingredient in the formulation. Moreover the derivative **SA1** will be more easily absorbed across biological membranes, thus increasing the bioavailability at the level of the target site.



Scheme 6. *Acetilation of Salicin.*

3.3.3 Antioxidant activity

The two natural compounds Baicalin and Salicin and the two semisynthetic derivatives **(B1** and **SA1)** were assayed to determine the antioxidant capacity (Table 12). ORAC test was used to measure their antioxidant capacity.

The method had been developed in order to evaluate the protection that antioxidants provide against OH and reactive peroxides, in particular for testing human serum samples, plant products, food and pharmaceutical ingredients. Unit of measure of the antioxidant power analyzed by this method is defined as ORAC units.

It is a very sensitive method that uses fluorescein as fluorescence marker (previously it was used the β-phycoerythrin less stable) and 2,2-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), azo compound soluble in water which decomposes thermally leading to the formation at a constant speed of aqueous peroxyl radicals.

In each sample, the inhibition of the degradation of fluorescein caused by the peroxyl radicals, thanks to the protective action of the antioxidants present, is a measure of the antioxidant capacity of the sample thereof with the reactive species. In fact if the test molecule is an antioxidant, it protects the fluorescence from the decay markers; terminated the effect of antioxidants, the radicals react with fluorescein leading to a decrease in fluorescence. The decay time of the fluorescence is proportional to the amount and the activity of the antioxidants present in the sample.

On a molar basis, the marker is chosen since this reacts with the oxygen radicals over 100 times more slowly than most of the organic antioxidants such as thiols, uric acid, bilirubin and ascorbate. Therefore, all other active antioxidants are completely oxidized before it is fluorescein, thus facilitating the measurement of the antioxidant capacity of the sample.

It is equally important that the marker is much more responsive to other non-antioxidant molecules, so it does not interfere in the analysis.

Each reaction, that occurs during the test, is calibrated using the standard Trolox®. The assay results are reported on the basis of equivalence:

1 unit ORAC = 1 µM of equivalents of Trolox®

Baicalin, Salicin and their semi-synthetic derivatives had been tested by ORAC test.

Compounds	**ORAC µmolTE/g**
Baicalin	5309.45 ± 2.3
B1	620.86 ± 1.4
Salicin	53.84 ± 1.2
SA1	28.52 ± 1.8

Table 12. *ORAC analysis of Baicalin, **B1**, Salicin and **SA1**.*
Each value was obtained from three different experiments (mean ± SE).

Baicalin is known to have a high antioxidant capacity, as confirmed by ORAC analysis, in which it had an activity of radical scavenger equal to 5309.45 µmol TE/g. For the evaluation of antioxidant activity of semisynthetic derivative **B1**, being chemically more

lipophilic than natural precursor, we adopted a different ORAC test, specific for lipophilic compounds. This test did not use PBS, solvent in which **B1** could precipitate during the time of analysis.

B1 had a very low antioxidant power (620.86 μmolTE/g) considering the chemical modification made.

Salicin and its derivative **SA1**, both showed no antioxidant activity against peroxide radical (53.84 and 28.52 μmolTE/g respectively). This result was supported by literature data that showed antioxidant activity *in vivo* of the natural molecule Salicin by breakage of glycosidic bond with the phenolic OH freeing. This, *in vitro*, in the ORAC method might not occur.

3.3.4 Antifungal activity

In order to verify the possible antifungal activity of natural compounds, Baicalin and Salicin, and their semi-synthetic derivatives **B1** and **SA1**, were tested on eight dermatophyte fungi (*paragraph. 3.1.5*).

		% Inhibition of growth	
Fungus		**Baicalin**	**B1**
Epidermophyton floccosum	20 µg/ml	+	1.6 ± 0.09
	100 µg/ml	0.00	11.1 ± 0.6
Trychopyton rubrum	20 µg/ml	+	+
	100 µg/ml	+	4.2 ± 0.6
Trichophyton tonsurans	20 µg/ml	+	1.4 ± 0.7
	100 µg/ml	0.00	13.9 ± 1.3
Tricophyton violaceum	20 µg/ml	+	11.4 ± 1.8
	100 µg/ml	+	20.0 ± 1.7
Tricophyton mentagrophytes	20 µg/ml	+	4.1 ± 0.5
	100 µg/ml	1.8 ± 0.1	6.6 ± 0.7
Mycrosporum gypseum	20 µg/ml	+	+
	100 µg/ml	+	+
Arthroderma cajietani	20 mg/ml	5.2 ± 0.7	7.2 ± 0.7
	100 µg/ml	7.5 ± 0.8	8.3 ± 0.9
Mycrosporum canis	20 µg/ml	2.4 ± 0.5	8.4 ± 1.2
	100 µg/ml	19.2 ± 1.1	18.9 ± 1.9

Table 13. *Percent growth inhibition of dermatophytes treated with Baicalin, and **B1** at 20 or 100 µg/mL. Each value is the mean of three measurements.*

As shown in Table 13, antifungal activity of Baicalin and its derivative **B1** did not result to be very interesting: in fact, in all cases the percentage inhibition values were quite low.

B1 was synthesized with the intention of making Baicalin more stable, however results of antimycotic activity seems to be more interesting.

The semi-synthetic derivative, in particular on *Trichophyton violaceum*, *Trichophyton tonsurans* and *Epidermophyton floccosum* showed higher inhibition values compared to its precursor, at concentrations of 20 and 100 μg/ml.

In all other cases it was observed a hormone-similar effect with growth of higher fungi compared to the control (+).

This result was so far unexplained and needed further investigation on the possible mechanism of action: **B1** was considered a pro-drug of Baicalin and should act by the same mechanism.

		% Inhibition of growth	
Fungus		**Salicin**	**SA1**
Epidermophyton floccosum	20 μg/ml	+	0.00
	100 μg/ml	+	5.1 ± 0.9
Trychopyton rubrum	20 μg/ml	+	0.8 ± 0.07
	100 μg/ml	+	4.9 ± 0.3
Trichophyton tonsurans	20 μg/ml	+	+
	100 μg/ml	0.00	+
Tricophyton violaceum	20 μg/ml	1.6 ± 0.2	2.1 ± 0.1
	100 μg/ml	1.6 ± 0.4	7.3 ± 1.7
Tricophyton mentagrophytes	20 μg/ml	10.6 ± 0.1	+
	100 μg/ml	22.7 ± 0.1	5.6 ± 0.7
Mycrosporum gypseum	20 μg/ml	+	24.3 ± 1.2
	100 μg/ml	+	32.1 ± 0.3
Arthroderma cajietani	20 mg/ml	+	+
	100 μg/ml	+	+
Mycrosporum canis	20 μg/ml	0.00	+
	100 μg/ml	5.3 ± 0.8	+

Table 14. *Percent growth inhibition of dermatophytes treated with Salicin, and **SA1** at 20 or 100 μg/mL. Each value is the mean of three measurements.*

Salicin and its semisynthetic derivative **SA1** did not have an important antifungal activity (Table 14); as in the previous case, the percentage inhibition values were rather low, except for Salicin that, on the fungus *Trichophyton mentagrophytes*, showed inhibition values equal to 10.6% and 22.7%, respectively, at concentrations of 20 and 100 μg/ml and for semi-synthetic derivative **SA1** that fungus *Microsporum gypseum*, gave an inhibition equal to 24.3% and 32.1% at the two concentrations.

As for the previous experiment, in all other cases it was observed a hormone-similar effect with higher growth of fungi compared to the control (+).

3.3.5 Salicin-PMMA complex

Natural and/or synthetic polymers are widely used in all industry, including pharmaceutical and cosmetic industries due to their versatility and practicality [75.76.77].

In particular, we chose to use as micro particles, polymethylmethacrylate (**PMMA**). It is a stable polymer, biocompatible with high mechanical resistance [78]. Among its many uses, it is used in the form of micro-particles in cosmetics [79], chromatography [80], pharmaceuticals (as a means of drug delivery) [81] and as a medical device.

Using emulsion technique [82] we tried to load Baicalin and Salicin in the **PMMA** microspheres. In particular, for Salicin, we used three **PMMA** (**PMMA-H**, **PMMA-P**, and **PMMA-P**) with different particle size and absorption capacity as shown in Table 15.

Polimer	***Particle size (μm)***	***Absorption capacity***
PMMA-H	8-12	3.0~4.0
PMMA-PH	8	2.1~2.4
PMMA-P	8	1.7~2.1

Table 15. *Chemical and physical characteristics of different types of PMMA.*

The quantity of Salicin that had been loaded in each polymer was tested by UV spectroscopy, using the calibration curve method with external standard (Figure 7).

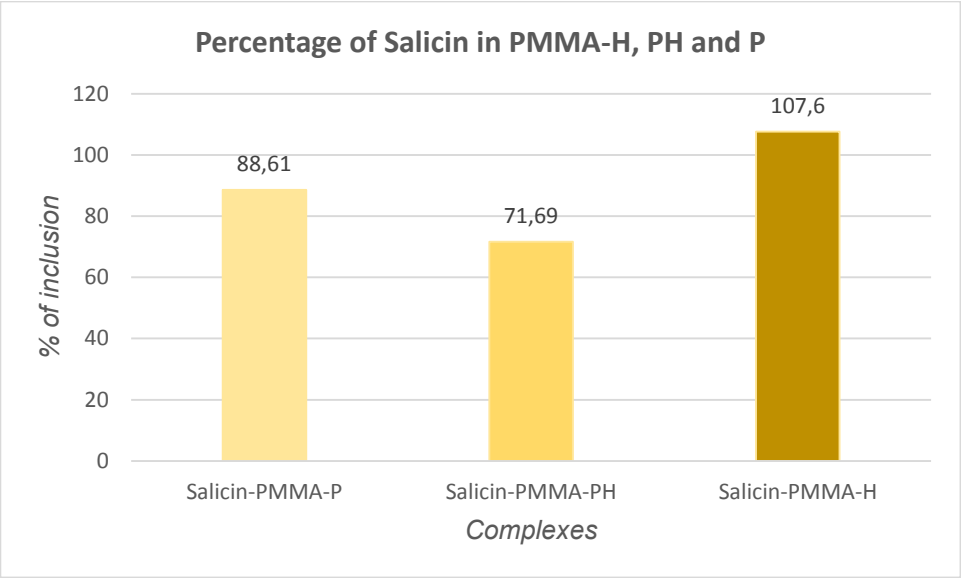


***Figure** 7. Percentage of Salicin's inclusion in PMMA-H, PMMA-PH and PMMA-P.*

PMMA-H, **PMMA-PH** and **PMMA-P** showed a high inclusion efficiency: **PMMA-H**, which was the best from an absorption point of view, seemed to be the polymer which had a greater power of inclusion (107.6%). Despite **PMMA-PH** having intermediate capacity between the two, it was the polymer which gived lower values as a percentage of loading.

3.3.5.1 Dissolution test (DT) on Salicin-PMMA complexes

Each of three complexes obtained, it was assayed by dissolution test. It is a procedure recognized by the European Pharmacopoeia for the *in vitro* release (for oral dosage forms) assessment of a drug that provides preliminary data on the behavior of a drug or a drug delivery system to the physiological level, mimicked by a solution at different pH ranges.

We used DT to understand if molecule was inside polymer.

As dissolution medium for each of the three tests a Phosphate Buffer at pH 7.4 was used. As proof that Salicin was soluble within the buffer, it was performed the same test also on the free active substance (Figure 8).

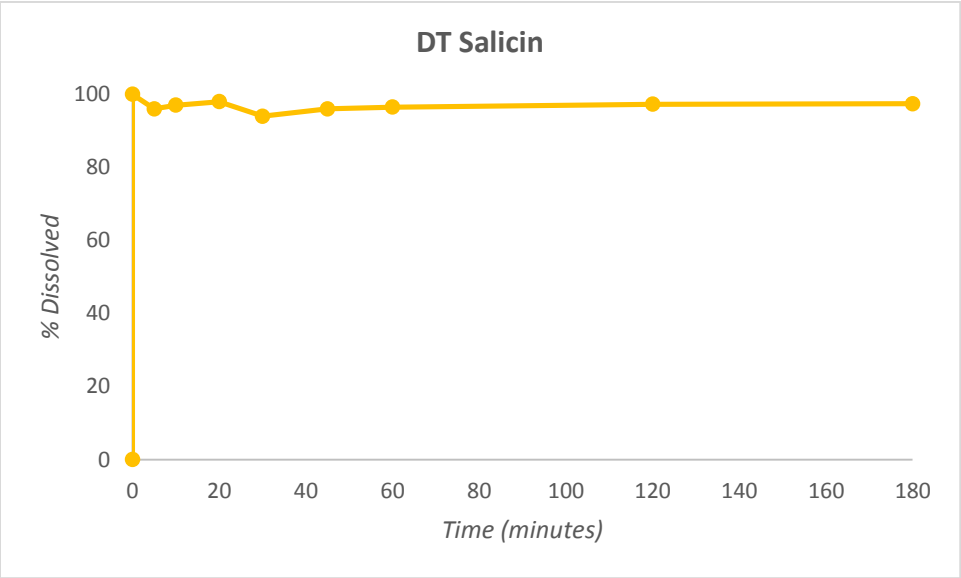


Figure 8. *DT of Free Salicin.*

From the data obtained, it was clear that Salicin was completely soluble in the medium used, in fact, already at T0 we get a dissolution of 100%.

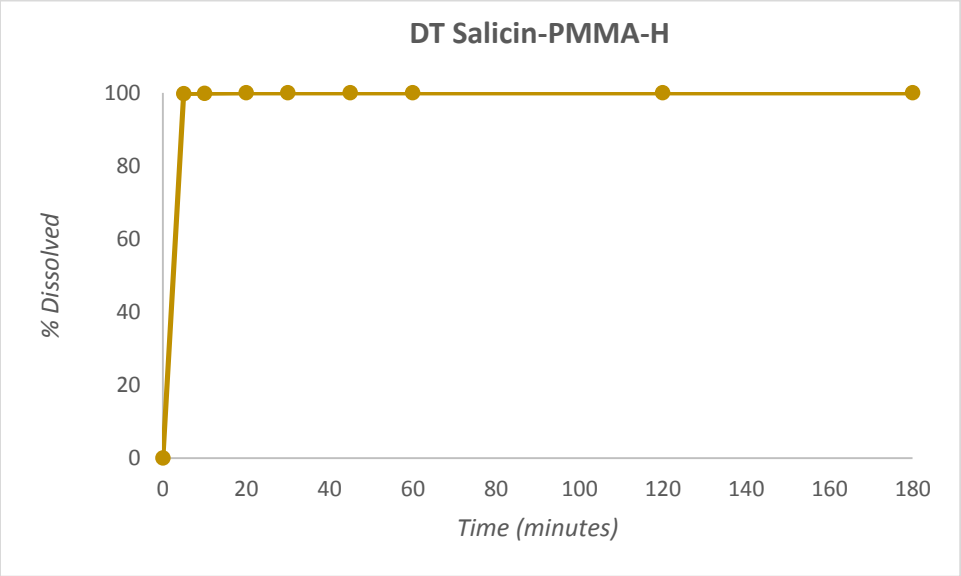


Figure 9. *DT of Salicin-PMMA-H.*

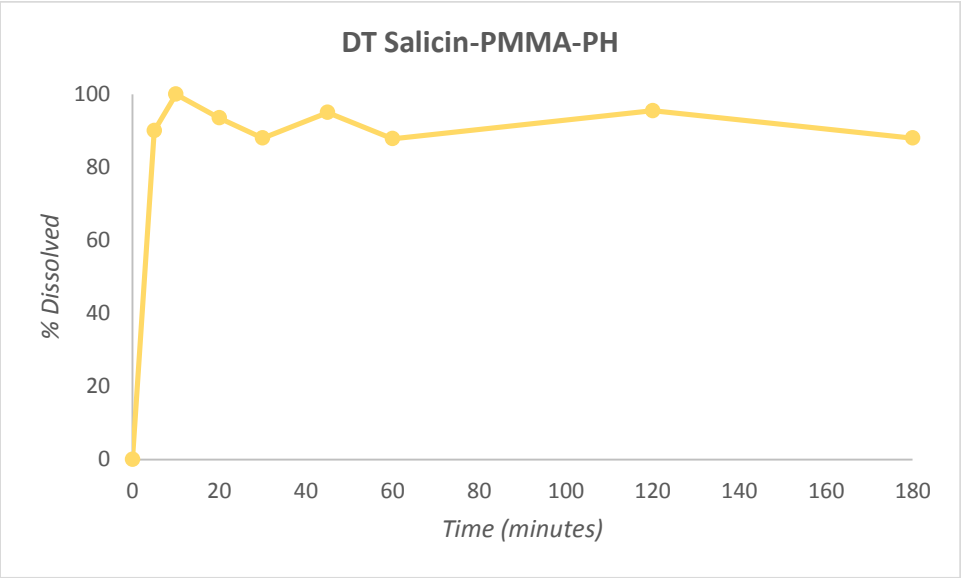


Figura 10. *DT Salicin-PMMA-PH.*

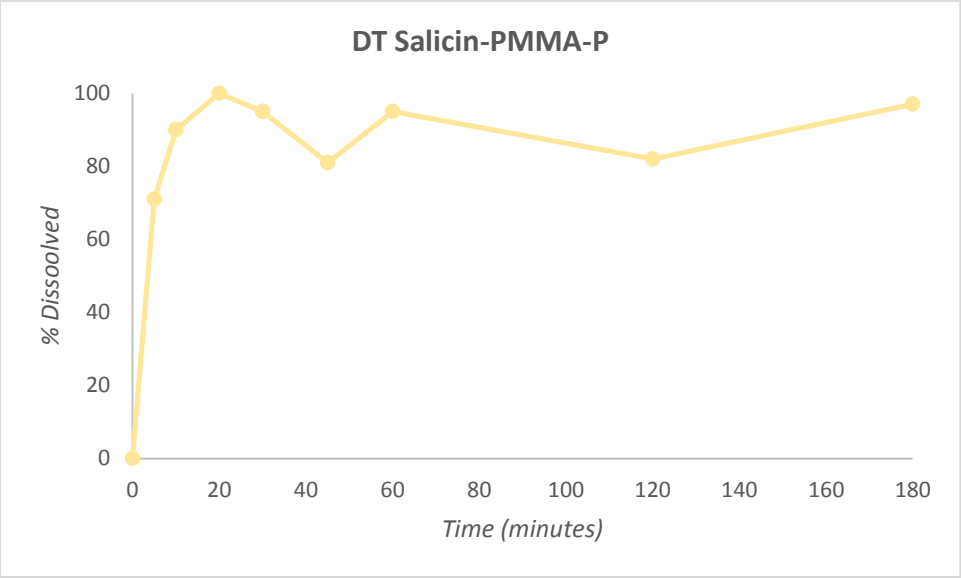


Figura 11. *DT Salicin-PMMA-P.*

DT results showed that there was a different release of active ingredient by the complexes compared to the free Salicin in the same dissolution media. In particular, **PMMA-H** showed fastest release (Figure 9), in fact, after only 5 minutes 100% Salicin was already dissolved in the buffer media. **Salicin-PMMA-P** complex (Figure 11) and **Salicin-PMMA-PH** (Figure 10) had reached 100%, respectively, after about 10 and 20 minutes.

DT was not carried out as a measure of controlled release but as proof that Salicin was inside the polymer and was not simply mixed with it or attaches on the polymeric matrix.

Therefore we considered these results positively, leaving the intention to continue the studies on the polymer performances.

For this purpose, since there were no big differences between the three selected **PMMA**, both in terms of release that loading, **PMMA-H** was selected for further studies.

3.3.6 Baicalin-PMMA-H complex

We decided to try the inclusion of Baicalin in **PMMA-H**. The choice of polymer for this experiment came from the fact that **PMMA-H** was, among those considered, the polymer with greater absorption capacity.

The analysis for the evaluation of inclusion capacity of **PMMA-H** was carried out by UV spectroscopy, using the calibration curve method with external standard.

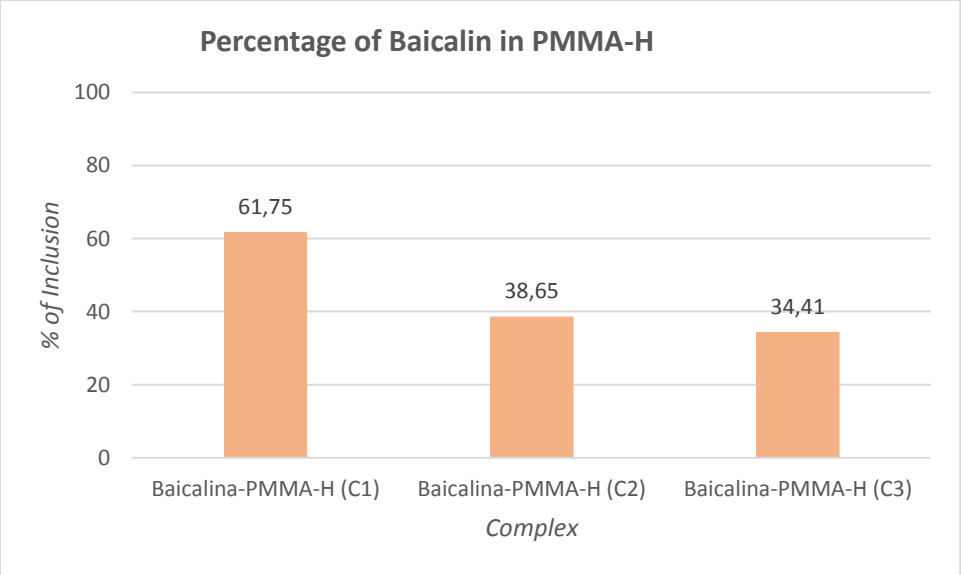


Figure 12. *Percentage of Baicalin loads into PMMA-H.*

Inclusion capacity of **PMMA-H** was lower in the case of Baicalin respect to Salicin. Probably this difference depended on different hydrophilicity of the test molecule, in addition to its greater steric hindrance.

3.3.6.1 Dissolution test (DT) on Baicalin-PMMA-H

Baicalin-PMMA-H complex was analyzed by **DT** using as dissolution medium phosphate buffer at pH 7.4.

Also in this case, **DT** was performed on free Baicalin in order to verify its solubility in the solvent of analysis (Figure 13).

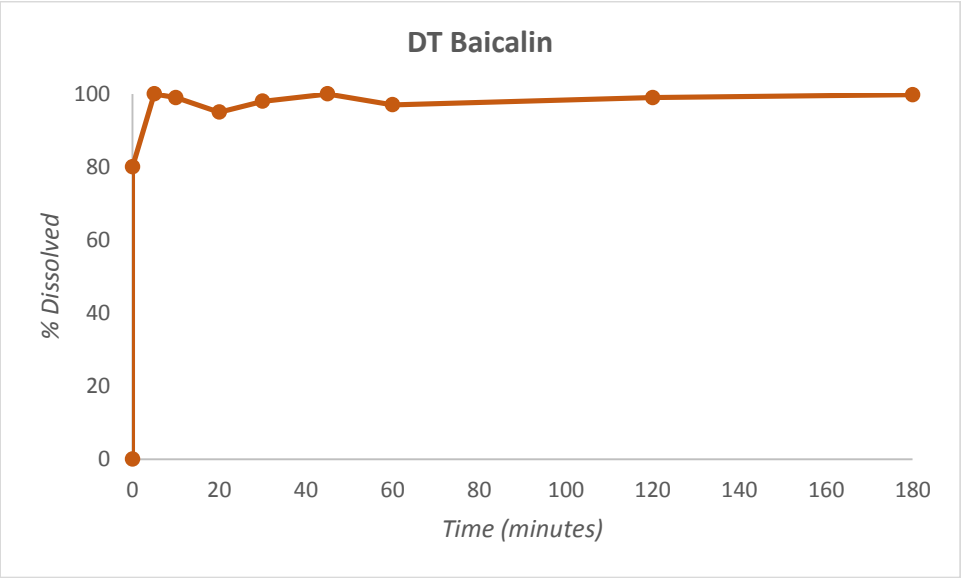


Figure 13. *DT Baicalin.*

As can be seen from Figure 13, Baicalin was very soluble in phosphate buffer, with a percentage of about 80% at T0.

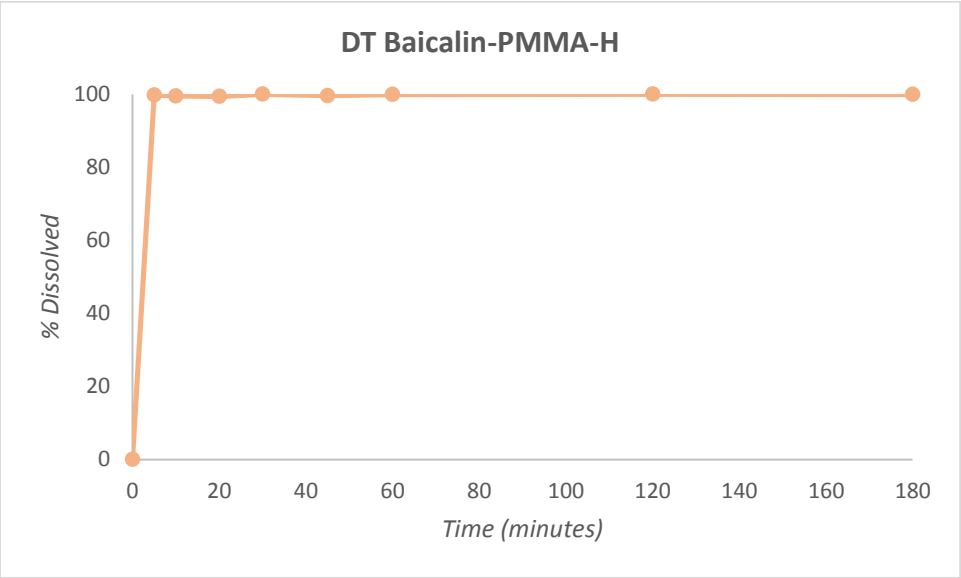


Figure 14. *DT Baicalin-PMMA-H.*

Baicalin-PMMA-H complex, released all Baicalin in Buffer solution after 5 minutes (Figure 14).

The data obtained from this inclusion suggested that the active ingredient was within the polymer. These results led us to conduct a more rational study on the inclusion method.

3.4 Loading method studies

The second part of the PhD project was carried out at the R&D department of the company Angelini. During this phase the inclusion of active within the polymer was investigated in detail, as a result of the initial screening described in the previous paragraph.

A careful study on polymeric materials and the specific loading techniques to structures already in the form of polymer particle, has led us to conduct a study on the possible inclusion and release of active from **PMMA**.

Two different loading procedures were followed:

1. Loading method with Ethanol: Hydrophilic molecules could be loaded into the polymer by dissolving in alcohol. This technique consisted in suspending

the polymer in EtOH; an EtOH solution of drug was slowly added to this suspension. Then solvent was dried in order to obtain a powder.

2. Loading method with oil: lipophilic molecules were dissolved in oil and the obtained solution was left to absorb by the polymer.

The first part of the study focused on inclusion capacity of **PMMA**, using two drugs with different solubility: Benzydamine hydrochloride (**BZD**) and Ibuprofen (**IBU**).

BZD was been chosen for the first loading method while **IBU** was been chosen for the second one.

Selected polymer was **PMMA-H**.

3.4.1 Loading method in EtOH

We made two **BZD-PMMA-H** complexes with the same tecnique, but with different concentrations of **BZD**: 10% and 20% respectively. On the two complexes obtained it was made the title of the active to ensure the presence and the homogeneity of **BZD** concentration in **PMMA-H**.

The title of the **BZD-PMMA-H** 10% and 20% were carried out by UV spectroscopy, using the calibration curve method with external standard and the data obtained are shown in the graphs below.

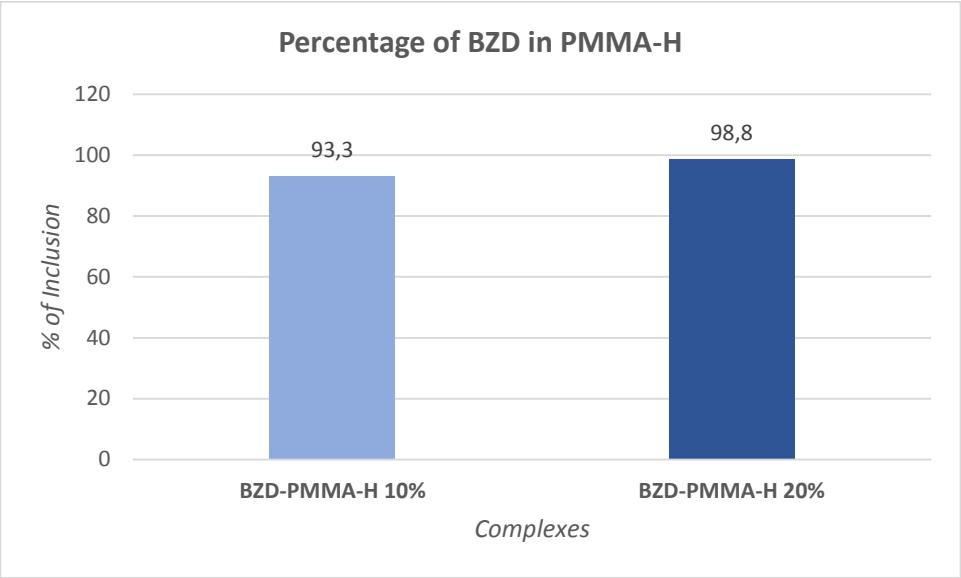


Figure 15. *Concentration of BZD in PMMA-H.*

As it can be seen in Figure 15, both for 10% and 20% loading were obtained good results, with more than 90% of **BZD** percentages.

Dissolution test was made on each complex in order to verify the release. Obviously, DT was not carried out as Pharmacopoeia essay but simply to verify that, even in high dilution conditions, polymer was capable of retaining the drug in its core. DT was also made with the intent to understand if **BZD** was within **PMMA-H**: a dissolution curve of free **BZD** and complexed one have confirmed this hypothesis.

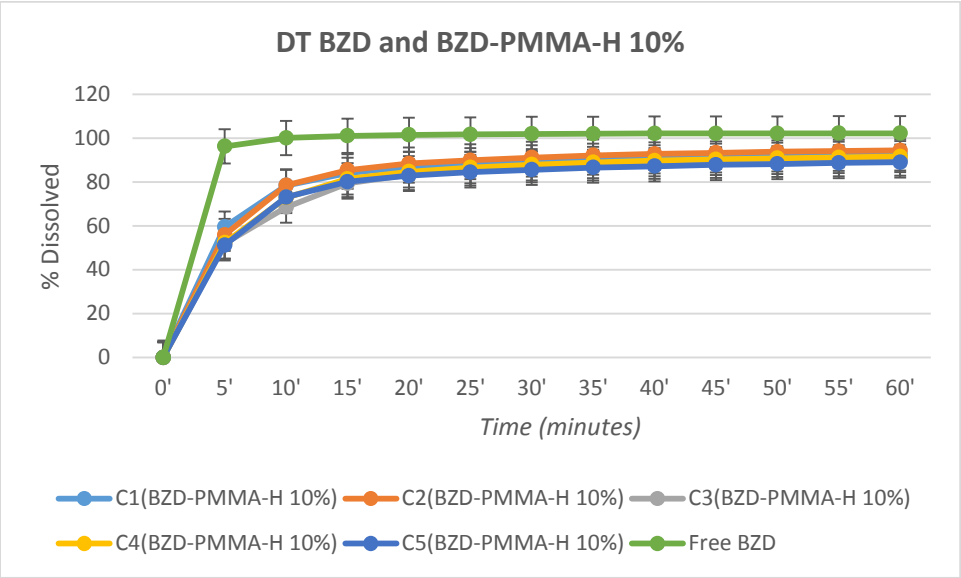


Figure 16. *DT of free BZD and BZD-PMMA-H 10%*

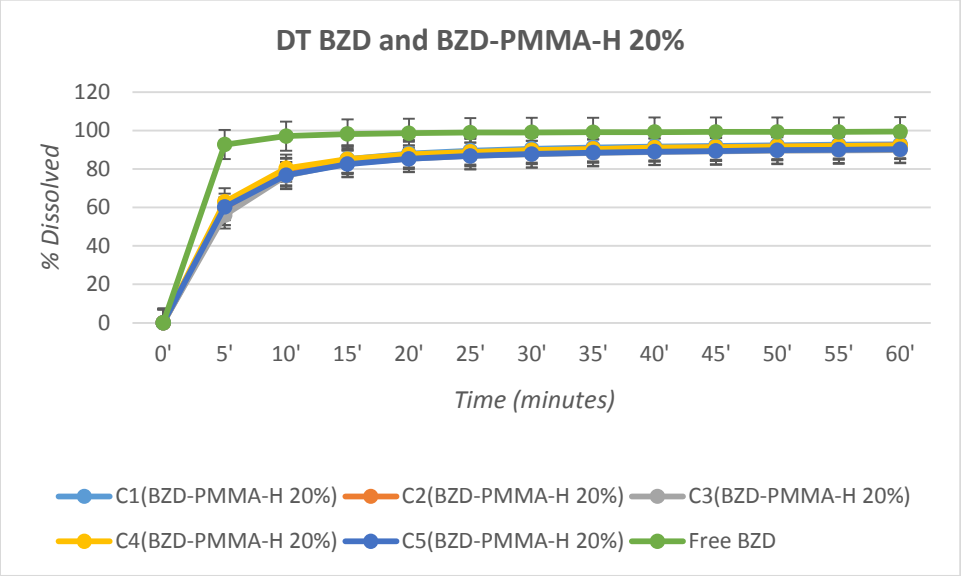


Figure 17. *DT of free BZD and BZD-PMMA-H 20%*

Figure 16 and 17 show dissolution curves of the two **BZD-PMMA-H** complexes. As you can see, each experiment was carried out on 5 random samples of each complex. Analyzing the data obtained we can say that the dissolution curves were similar each other and, in both cases plateau was reached after 20 minutes of analysis. On the contrary, 100% of free **BZD** was already found, in both analysis, after 5 minutes in the dissolution medium. **BZD-PMMAH 20%** complex appeared more uniform than **BZD-PMMAH 10%**.

3.4.2 Loading method in Oil

Two different oils were selected to be used for this loading method, a no volatile oil, that after the absorption remained into the polymer, and the silicon that after being absorbed by **PMMA-H** was able to evaporate.

Selected oils were:

- Vaseline
- Hexamethyldisiloxane (**HMDS**)

IBU-PMMA-H complexes were prepared using the two oils in which the drug was soluble.

We made two complexes:

1. **IBU-PMMAH 10%** e **20%** in Vaseline;
2. **IBU-PMMAH 10%** e **20%** in HMDS.

Figure 18-20 reported the percentage of **IBU** in **PMMA-H** and the results obtained by dissolution tests.

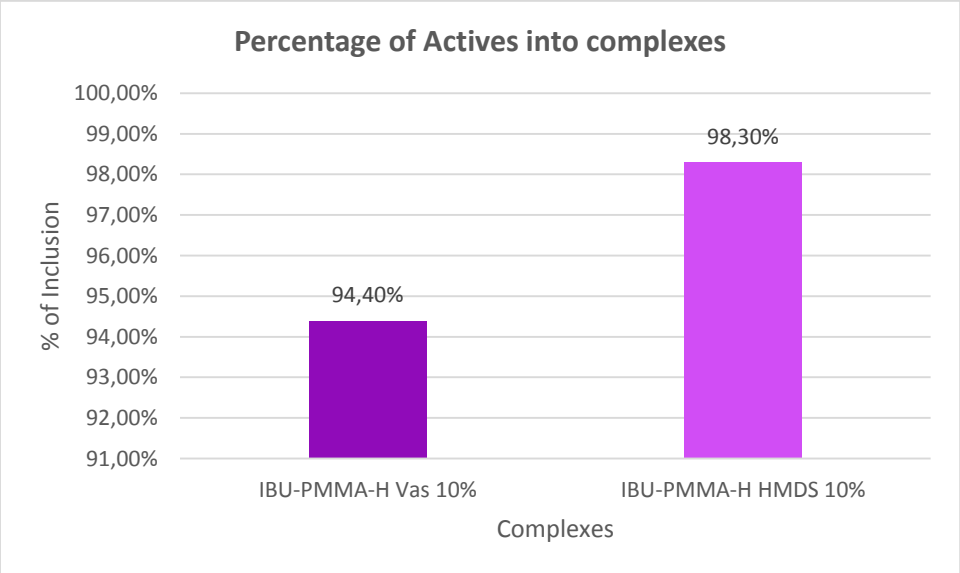


Figure 18. *Percentage of IBU in PMMA-H.*

As can be seen from the values obtained, percentage of **IBU** in the two complexes was congruent with the loading concentrations.

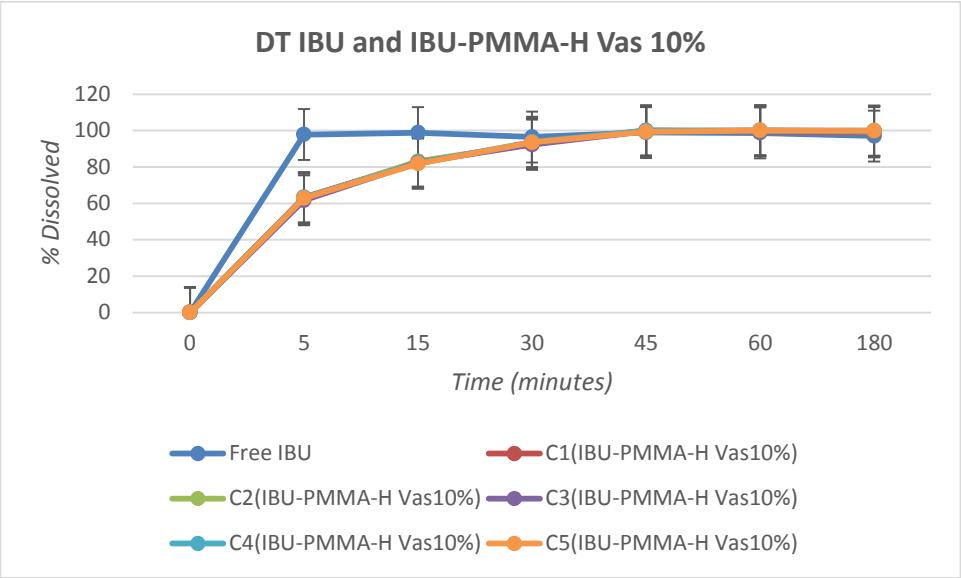


Figure 19. *DT IBU-PMMA-H 10% loading by Vaseline.*

DT release curves were done only for **IBU-PMMA-H 10%** (loaded with Vaseline and HMDS) because the loading method was the same both for 10% and 20% so their performances might be the same.

It is important to remember that DT was important for us to understand if the drug was inside the polymer core.

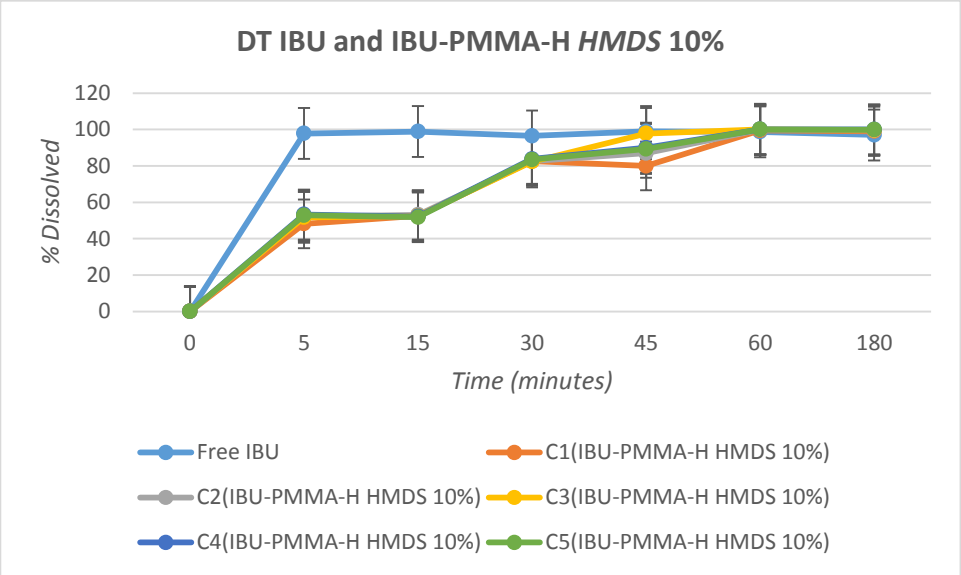


Figure 20. *DT IBU-PMMA-H loading by HMDS.*

For **IBU-PMMAH 10%** HMDS and Vaseline, 100% of drug was dissolved after 45 minutes of analysis. These data confirm that the release of the drug through the polymer was different if there was an oil phase.

In fact, for the Vaseline complex, the 70% of **IBU** was already dissolved after approximately 5 minutes, followed by 90% after 15 minutes, while in the case of the same complex in HMDS, the release of **IBU** after 5 minutes was only 50% followed by about 60% after 15 minutes.

At least in these conditions we were far away from what was the target of our complex: the skin.

Once validated the loading method, the objective was to check the release of the drug in the presence or not of the complex.

3.5 In vitro release study

Franz cell system is the most common test to study in vitro release (**INVR**) of drugs from semi-solid forms. To evaluate the kinetics of drug release from semi-solid formulations, there are guidelines that recommend the use of Franz cells with a membrane that is not a barrier, but only a support for the formulation [83.84].

The method is accepted by FDA for the scale up and for post-approval changes.

A typical vertical Franz cell system is shown in Figure 21: formulation is placed in the donor Superior compartment and it is separated from the receiving media by a membrane, which can be a synthetic membrane, a construct or biological tissue, deriving by the dead skin [86.87]. Diffusion of drug from the semi-solid product through the membrane is monitored by spectroscopic analysis (UV or HPLC, usually).

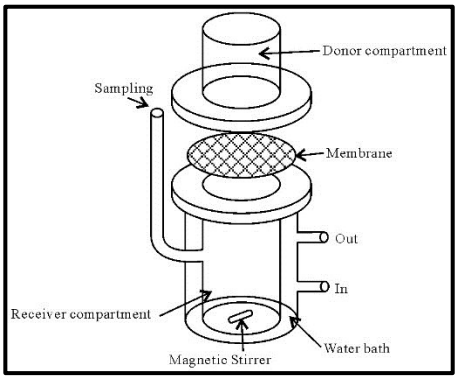


Figure 21. *Schematic rappresentation of Franz cell system.*

Each complex, in different formulations, was analyzed by Franz cells in order to compare the release with the same formulation containing free drug.

3.5.1 Topical formulations with BZD

In this study **BZD-PMMA-H** complex was added in two formulations: a hydrophilic gel, in which the drug is highly soluble and a cream, a pharmaceutical O/W emulsion, where **BZD** is less soluble. This choice was made to verify if the performances of the **PMMA-H** varied depending on the solubility of the drug within the formulation.

3.5.1.a Gel

Two Gels had been formulated: one with the free drug and the other one with **BZD-PMMA-H 20%** complex. Each of the two formulations had been analyzed the day of manufactured (T0) and after 30 (T30), 60 (T60) and 90 days (T90).

The results obtained are reported in Figure 22-25.

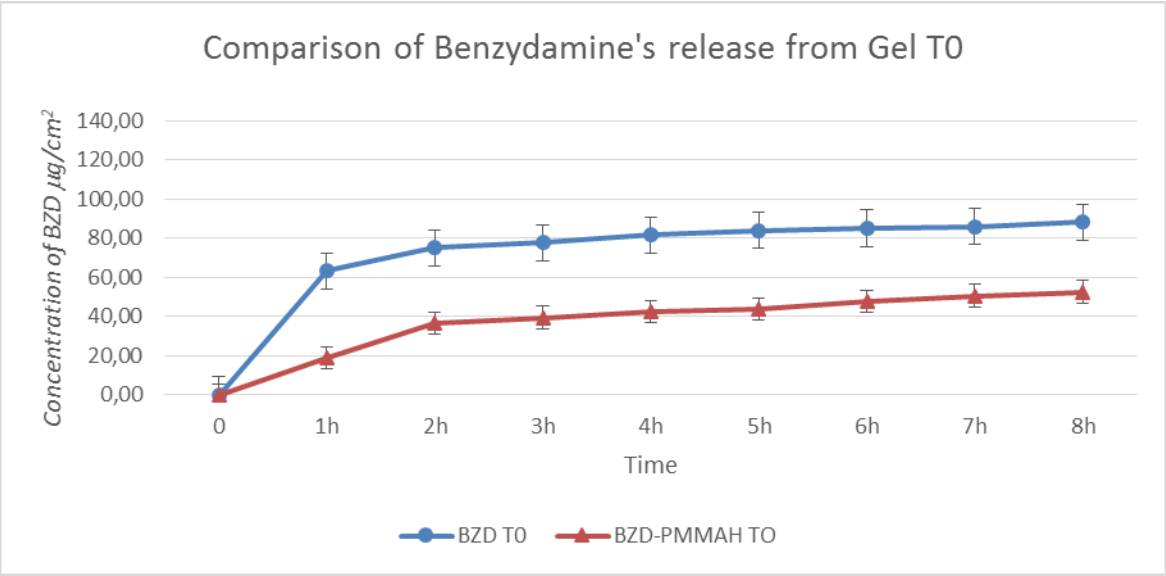


Figure 22. *BZD release from gel T0.*

As can be seen from the graph, the release of complexed **BZD** by the gel was less than 50% to the same gel with free BZD. Moreover, the difference in the release remained constant for the eight hours of analysis. This result confirmed the inclusion of **BDZ** in the polymer.

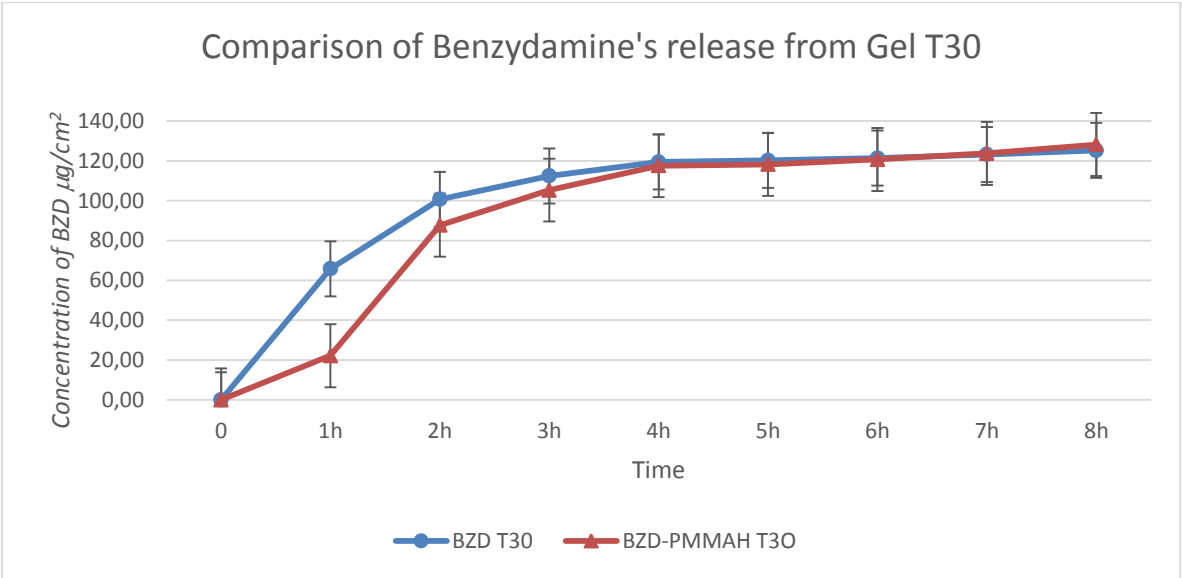


Figure 23. *BZD release from gel T30.*

After 30 days (Figure 23) the release of **BZD** increased from both formulations and the two curves were much closer to each other. In fact, the difference of release was 40% after an hour but after only four hours the two curves were alike.

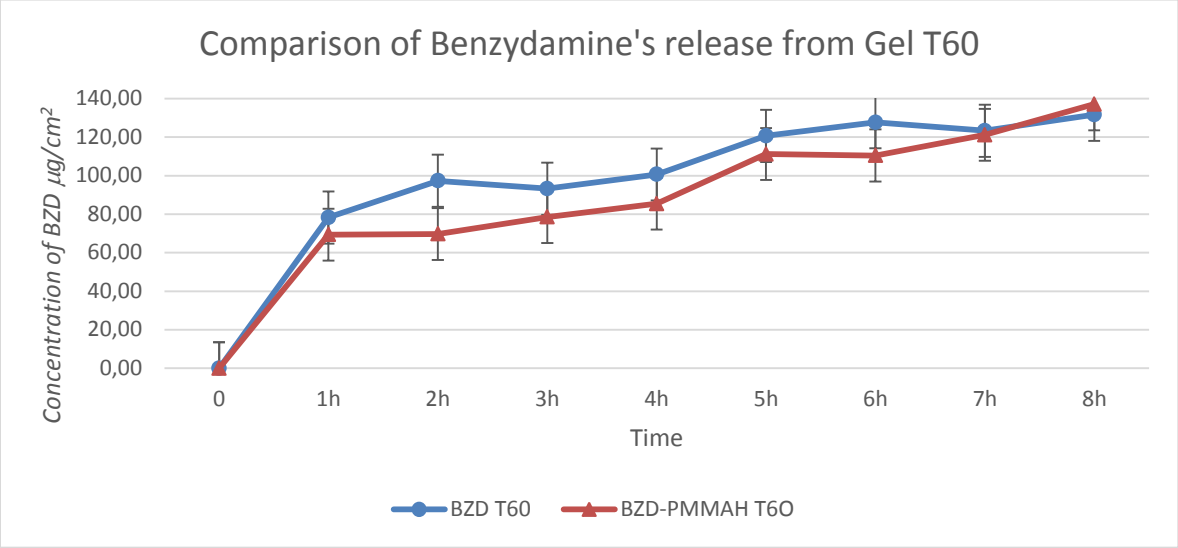


Figure 24. *BZD release from gel T60.*

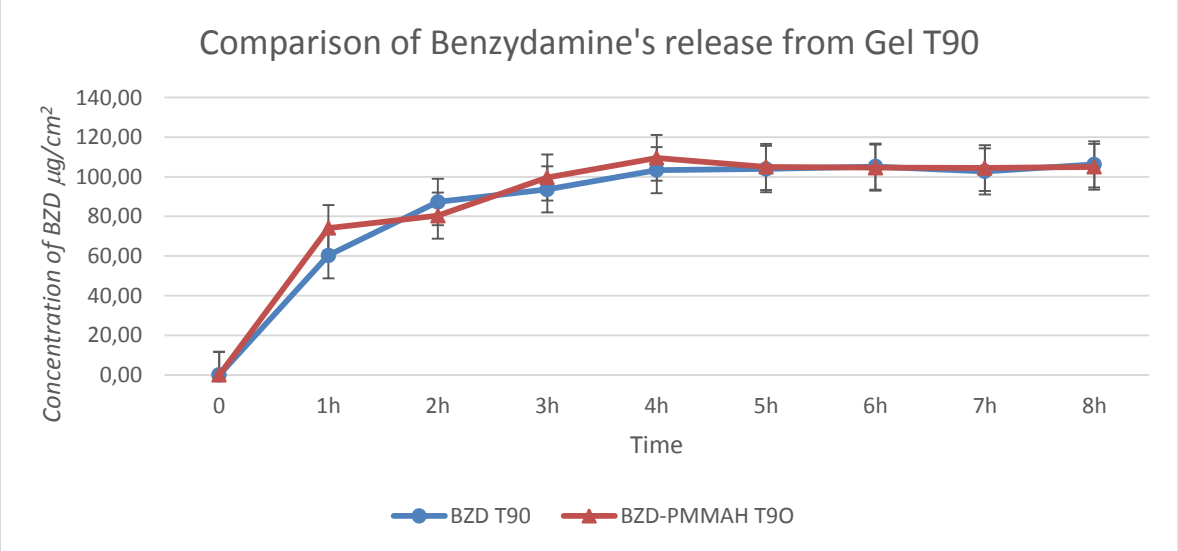


Figure 25. *BZD release from gel T90.*

The analysis after 60 and 90 days confirmed that the aqueous medium could probably spread into the polymer and the drug dissolved, that made the release of **BZD** from the complex equal to the formulation with free drug.

3.5.1.b Creams

The release analysis was performed on free **BDZ** and **BZD-PMMA-H**, in the same type of cream. Each of the two formulations had been analyzed the day of manufactured (T0) and after 30 (T30), 60 (T60) and 90 days (T90).

Data obtained are reported in Figure 26, 27, 28 and 29.

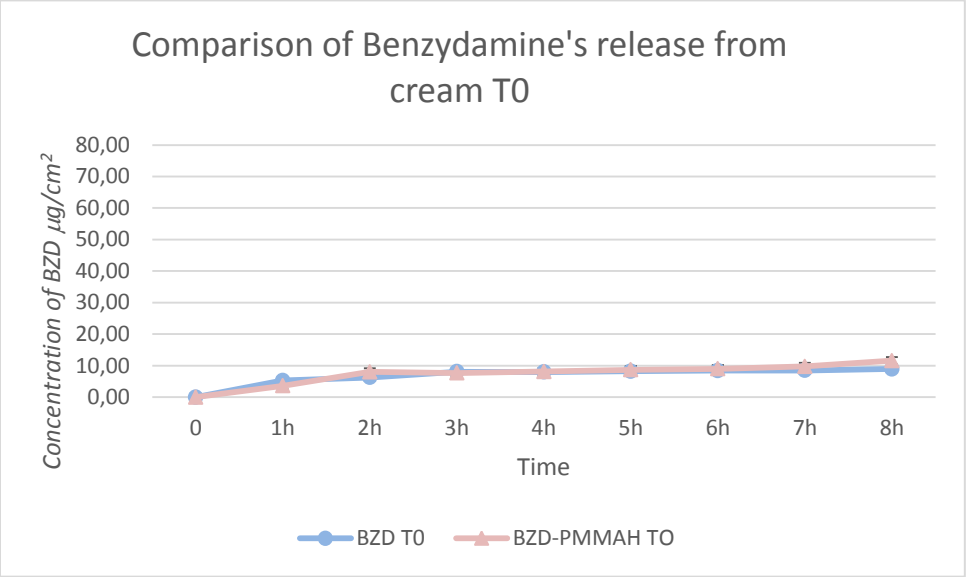


Figure 26. *BZD release from cream T0.*

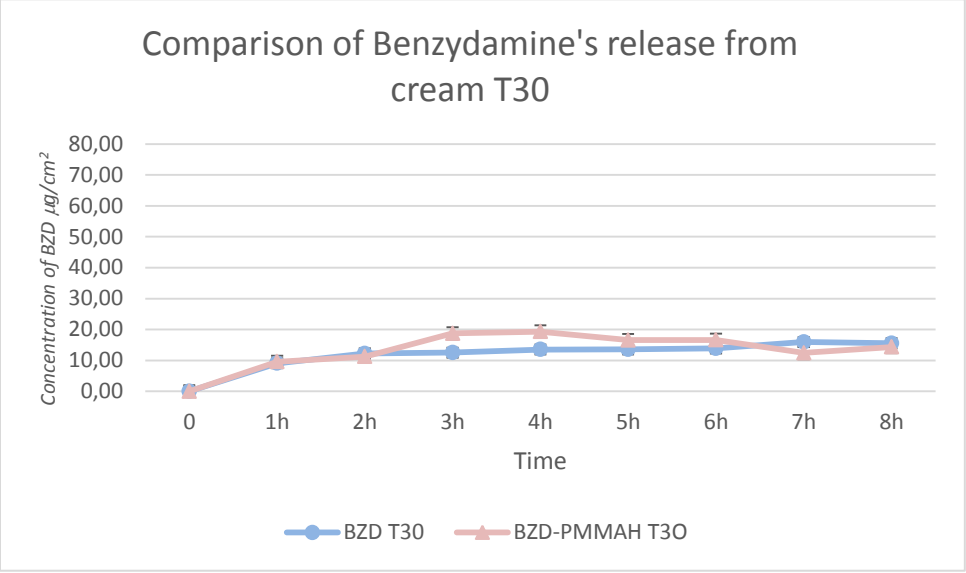


Figure 27. *BZD release from cream T30.*

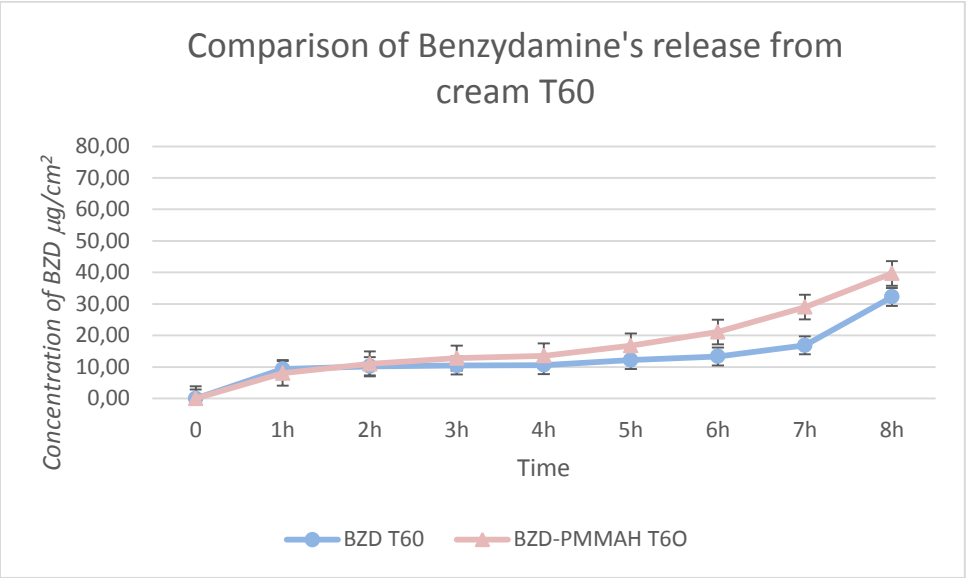


Figure 28. *BZD release from cream T60.*

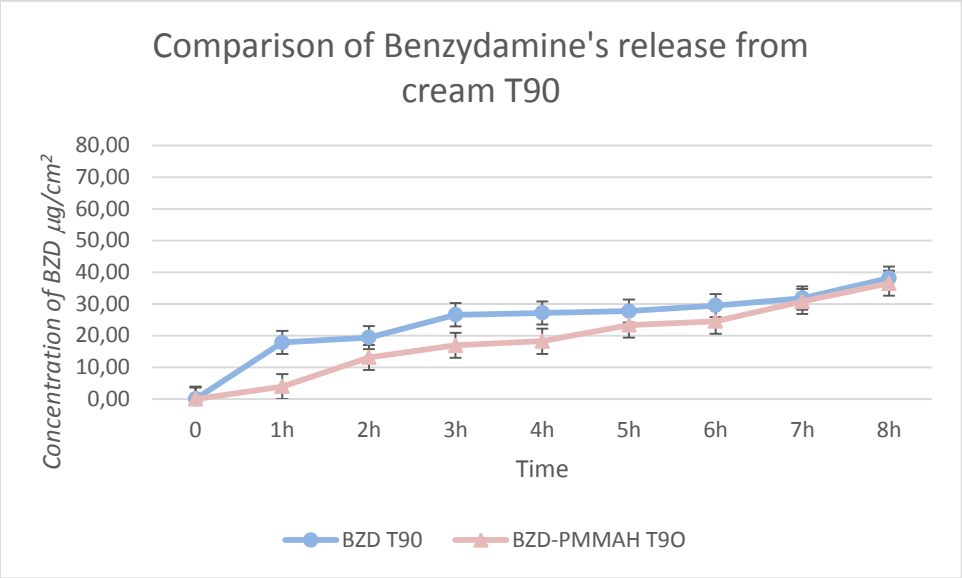


Figure 29. *BZD release from cream T90.*

In the case of cream, release curves were very similar each other already at T0, and the result was repeated until T90. Probably this event was due to the **PMMA-H** capacity to absorb oil: **PMMA-H** absorbed oil phase and released the drug in the formulation.

3.5.2 Topical formulations with IBUPROFEN

The release of Ibuprofen had been carried out using a lipogel with free and complexed drug (**IBU-PMMA-H)** obtained with two different oils, Vaseline and HMDS. Each of the three formulations had been analyzed the day of manufactured (T0) and after 30 (T30), 60 (T60) and 90 days (T90).

The release data at T0, T30, T60 and T90 are shown in Figure 30.

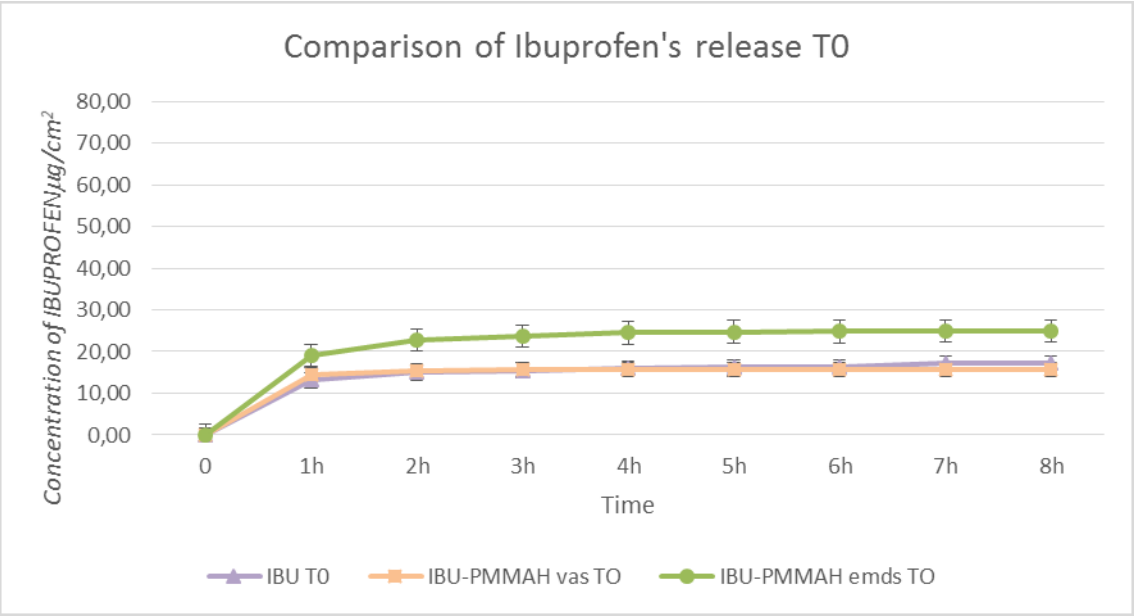


Figure 30. *IBU release from lipogel, T0.*

The data shows that **PMMA-H** was not able to hold the drug in the presence of oil phase.

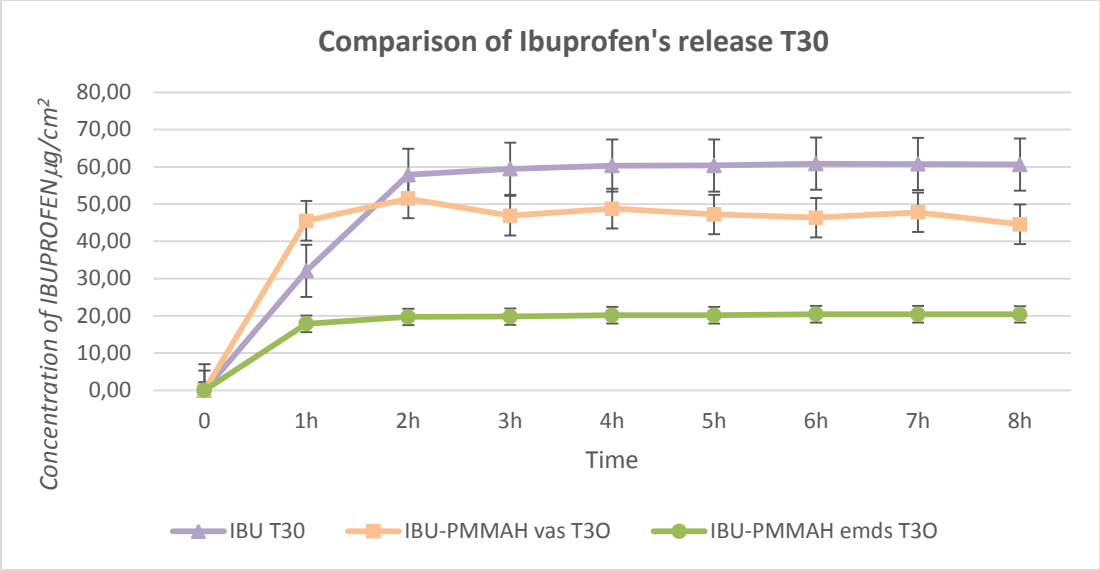


Figure 31. *IBU release from lipogel, T30.*

After 30 days, the release of Ibuprofen from the formulation with free Ibuprofen and **Ibuprofen-PMMA-H** loaded with Vaseline had increased by 50% compared to the first analysis. While, **Ibuprofen-PMMA-H** loaded with HMDS was still equal to T0.

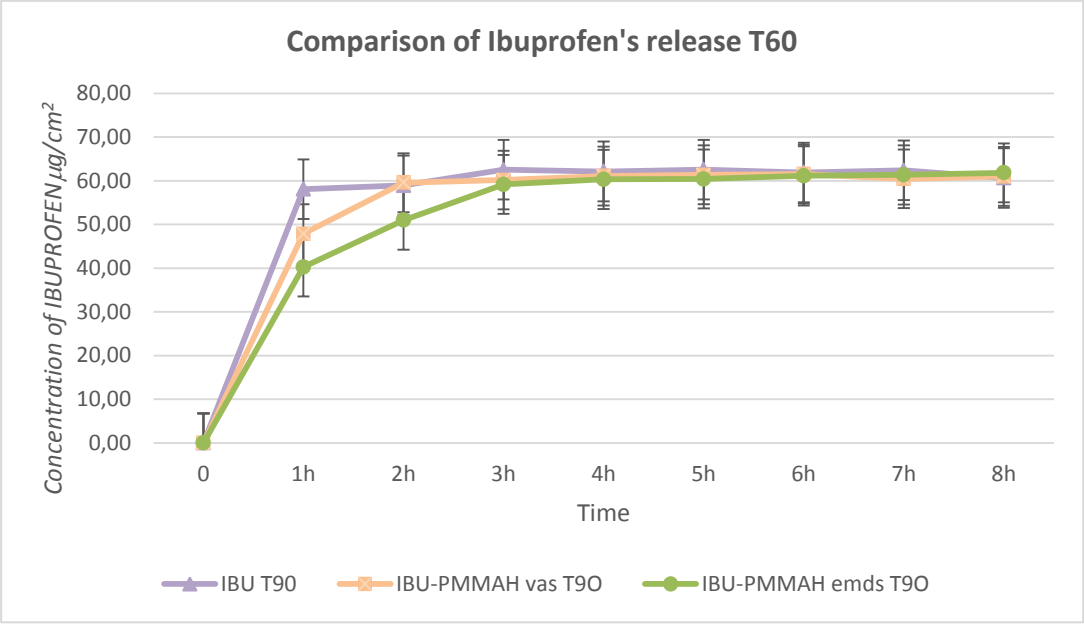


Figure 32. *IBU release from lipogel, T60.*

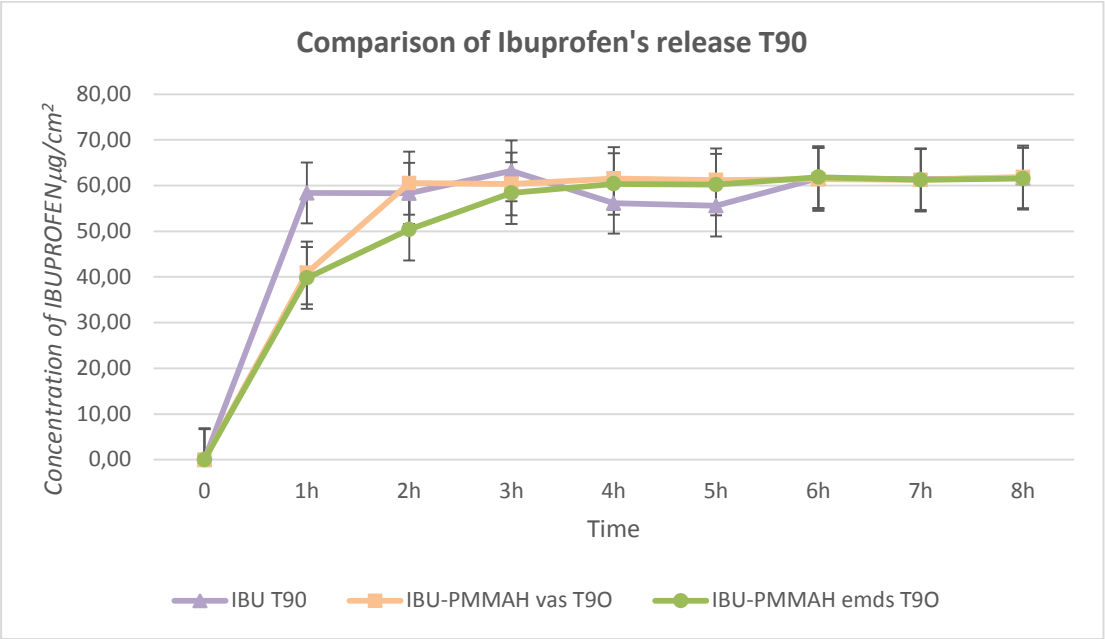


Figure 33. *IBU release from lipogel, T90.*

After 60 and 90 days, the release of drug from all formulations was the same (Figures 32 and 33).

3.6 Loading of cosmetic ingredients

At the same time to the study of drugs above, it had been studied the inclusion of two cosmetic active ingredients:

1. **18β Glycyrrhetic Acid (GLA),** active metabolite of glycyrrhizic acid (natural compound extracted from licorice), has a variety of pharmacological effects (anti-inflammatory, anti-allergic, anti-carcinogenic and anti-immune mediated) [88.89]; it has the ability to inhibit the emergence and growth of skin tumors [90.92], it also has skin protection activity, soothing and sebum control. **GLA** is also an anti-inflammatory and a radical scavenger molecule [93]. It accelerates wound healing and it is used in many skincare products (also baby products).

2. **Quercetin (QE)**, is the aglycone of rutin, presents in different plant species. From a cosmetic point of view the main activity of Quercetin is the antioxidant and UV protective activity [94].

GLA and **QE** were loaded into **PMMA-H** with the method described in *Paragraph 3.4.1,* and have been inserted in two different formulations (a W/O emulsion and an O/W emulsion). **GLA** and **QE** concentration in each complex had been verified by UV spectroscopy, using the calibration curve method with external standard and the data obtained are reported in the graph below.

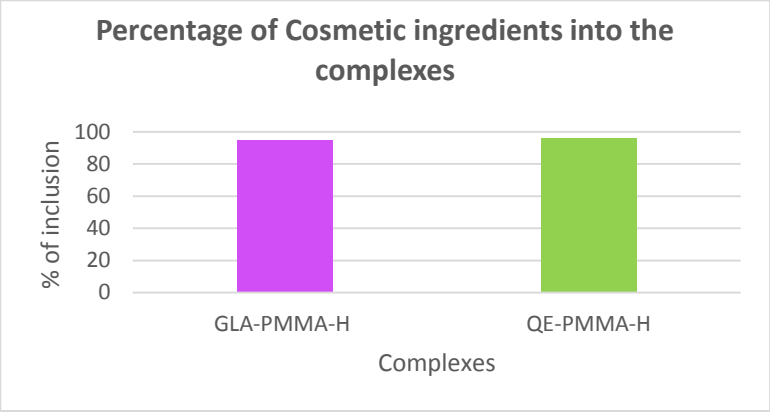


Figure 34. *Percentage of GLA and QE into the polymer.*

The two formulations containing **GLA-PMMA-H** had been studied using Franz cells while, the two formulations containing **QE-PMMA-H** had been investigated in terms of stability.

3.6.1 Release study on Glycyrrhetic Acid W/O emulsion

The release of **GLA** had been carried out using a W/O formulation with free active and **GLA-PMMA-H** complex obtained with ethanol technique. Each of the two formulations had been analyzed the day of manufactured (T0) and after 30 (T30), 60 (T60) and 90 days (T90).

The release data at T0, T30, T60 and T90 are shown in Figure 35-38.

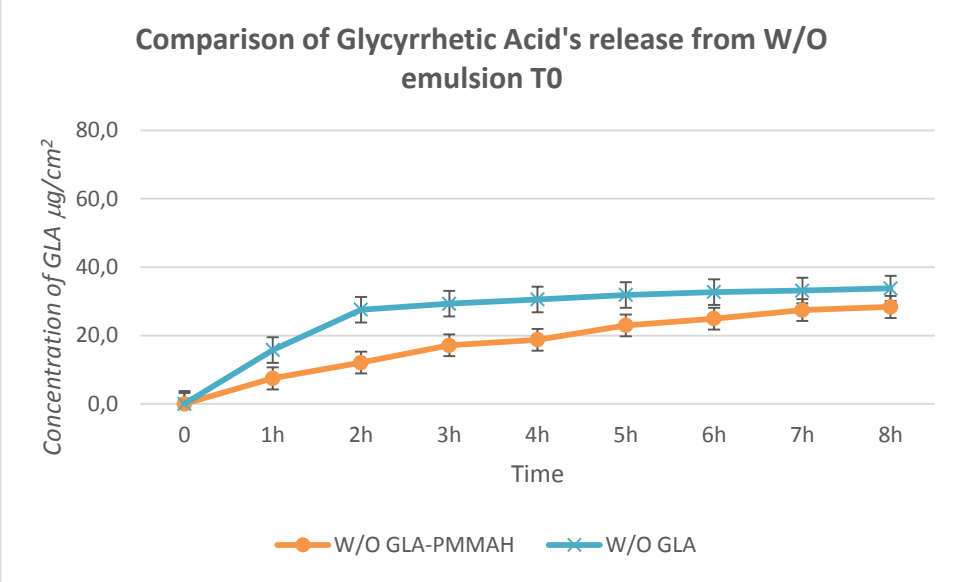


Figure 35. *Release of GLA from W/O emulsion at T0.*

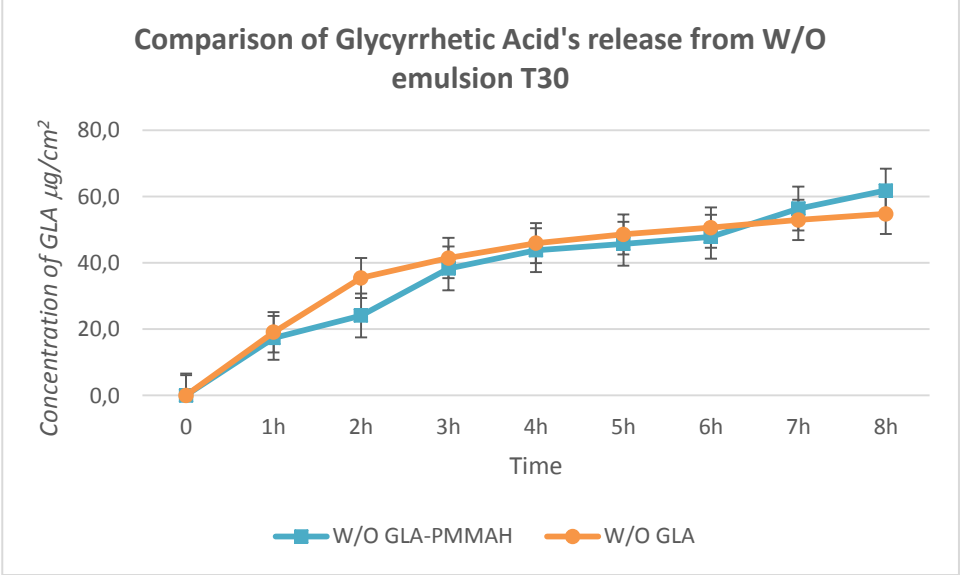


Figure 36. *Release of GLA from W/O emulsion at T30.*

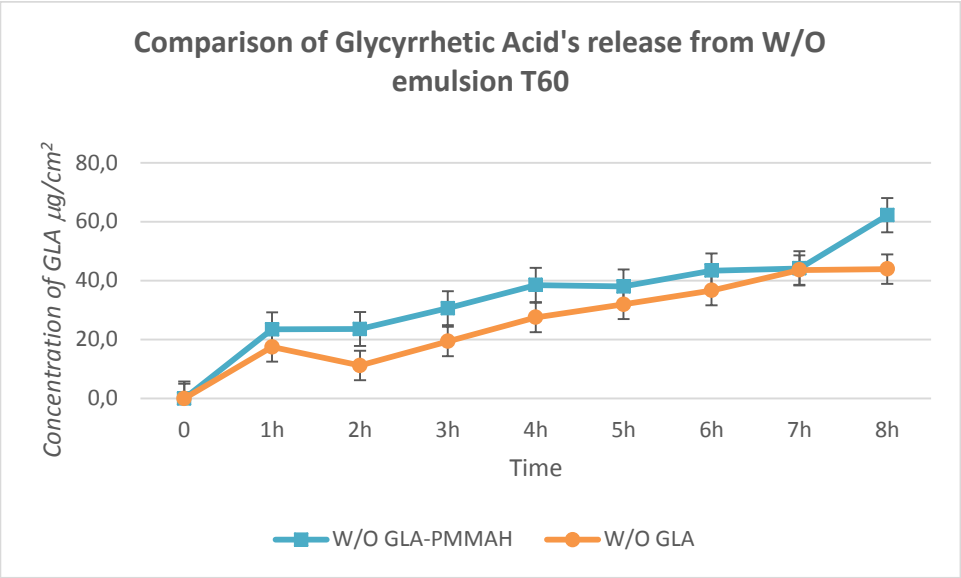


Figure 37. *Release of GLA from W/O emulsion at T60.*

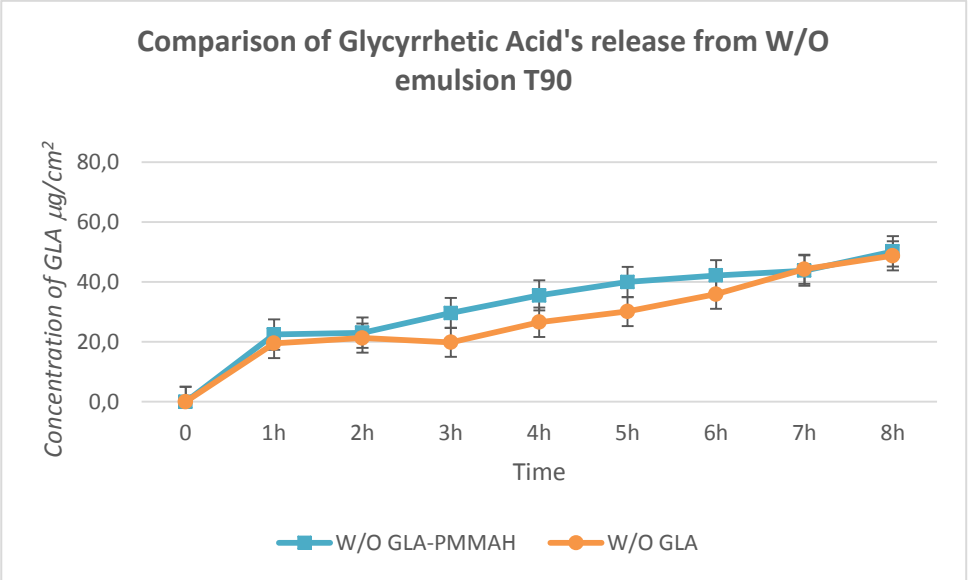


Figure 38. *Release of GLA from W/O emulsion at T90.*

As can be seen from the release curves obtained, a difference of release was only obtained by the analysis at **T0** (20%), subsequently, **T30**, **T60** and **T90** analysis show that the two curves were comparable each other.

3.6.2 Release study on Glycyrrhetic Acid O/W emulsion

The release of **GLA** had been carried out using an O/W formulation with free active and **GLA-PMMA-H** complex obtained with ethanol technique. Each of the two formulations had been analyzed the day of manufactured (T0) and after 30 (T30), 60 (T60) and 90 days (T90).

The release data at **T0**, **T30**, **T60** and **T90** are shown in Figure below.

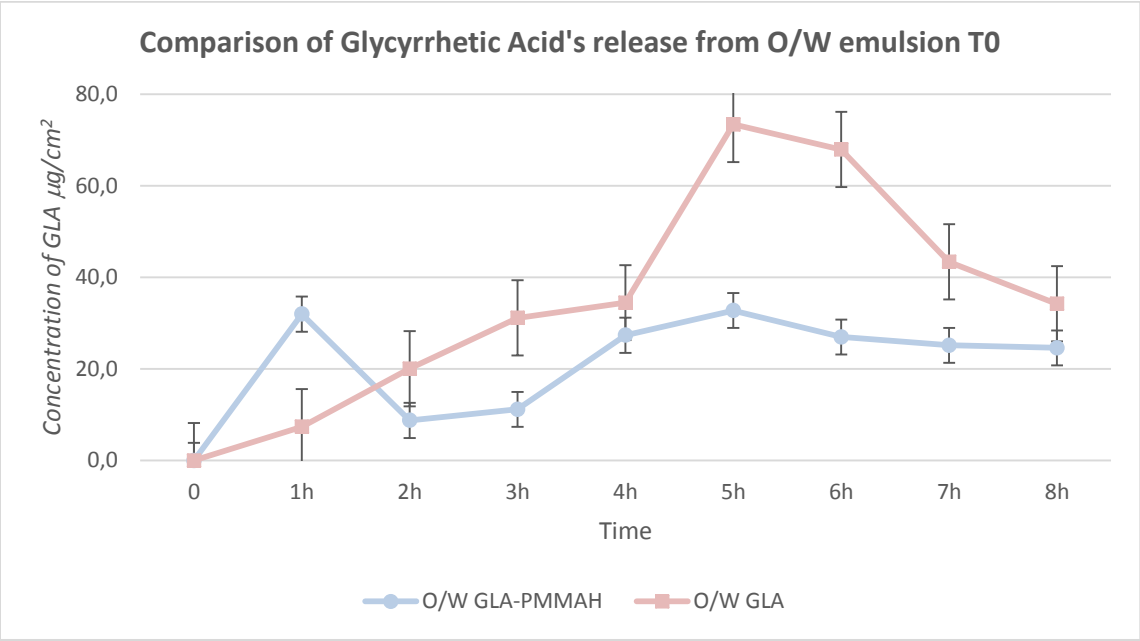


Figure 39. *Release of GLA from O/W emulsion at T0.*

Curves obtained at **TO** did not show a linear trend, probably cream needed a longer time to stabilize.

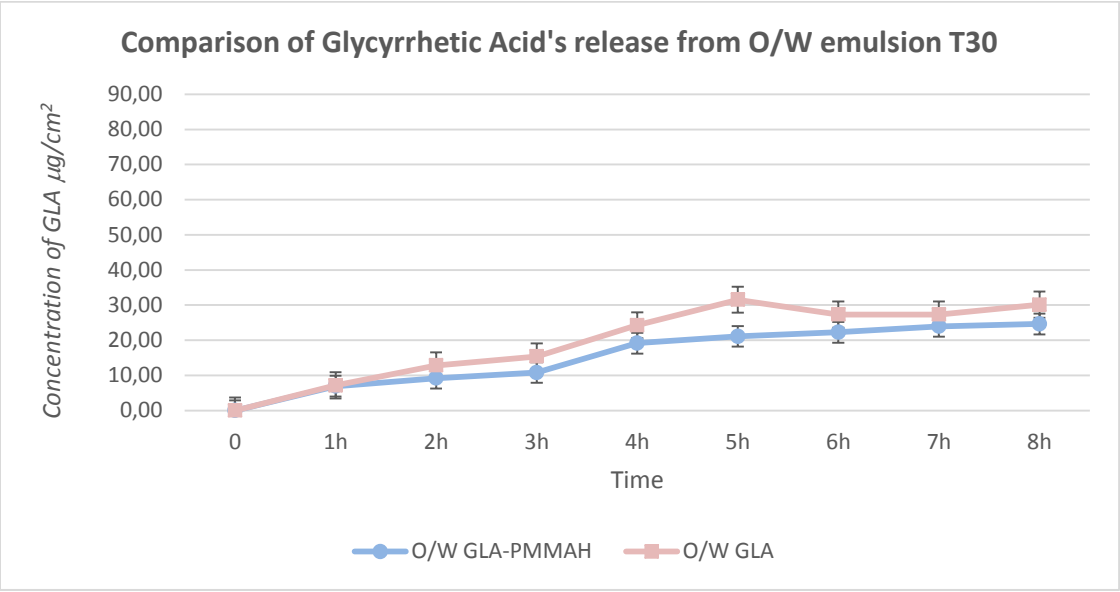


Figure 40. *Release of GLA from O/W emulsion at T30.*

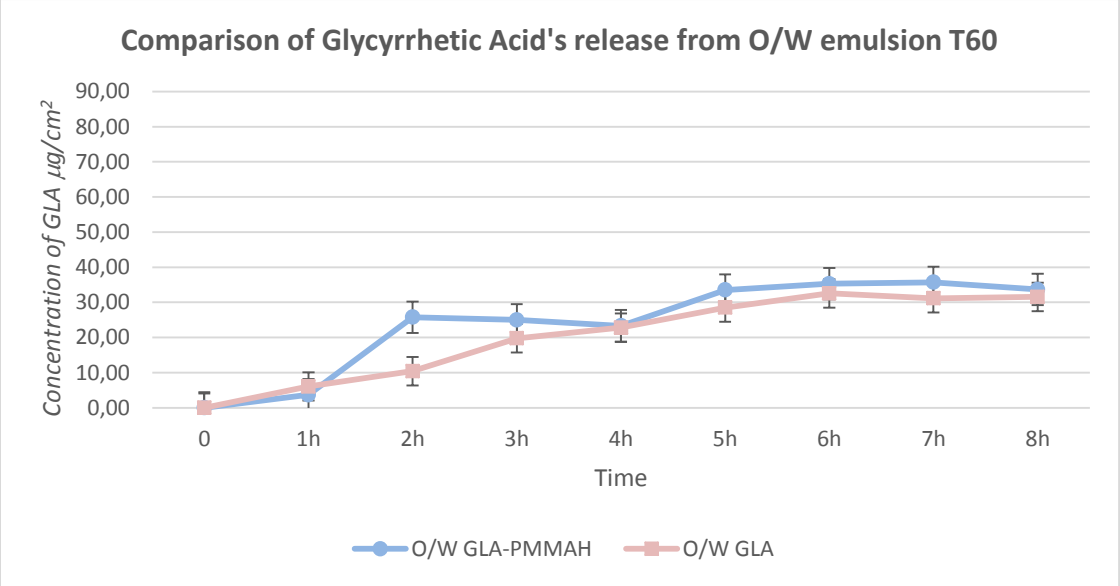


Figure 41. *Release of GLA from O/W emulsion at T60.*

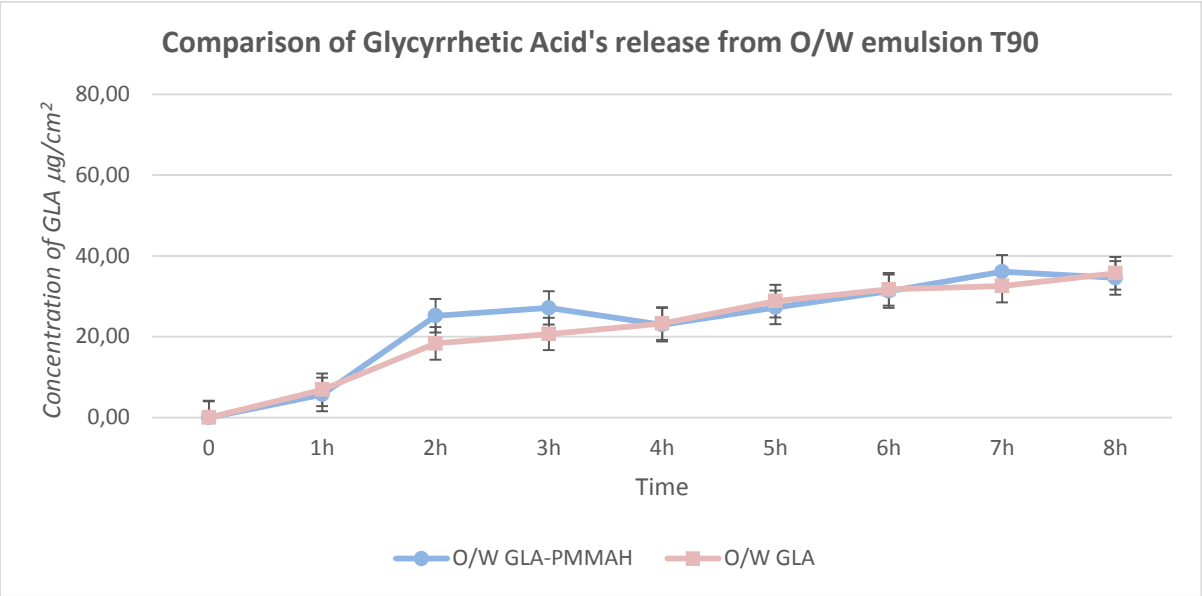


Figure 42. *Release of GLA from O/W emulsion at T90.*

Analysis made at **T30**, **T60** and **T90** had a normal pattern with very similar curves between free **GLA** and **GLA-PMMA-H**.

3.6.3 Study on topical formulation with Quercetin

The stability of the two formulations, W/O and O/W emulsion, containing as active ingredient **QE** had been studied.

Being quercetin a substance with known antioxidant capacity, we monitored this activity to assess the stability of **QE** free and complexed with **PMMA-H** in the same type of formulation. Antioxidant activity was monitored by PCL Test, according to accelerated stability test protocol (90 days at 40°C).

The result obtained are reported in Table 16.

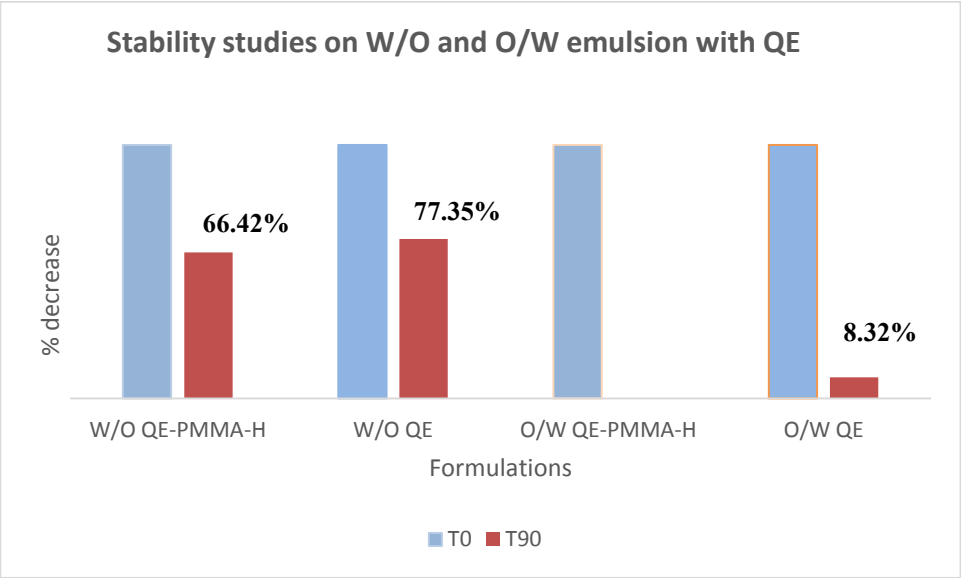


Table 16. *Decrease of Antioxidant activity of QE in different formulations.*

QE was found to be less stable in the emulsion W/O rather than in the O/W. In each case, W/O emulsion that containing **QE** complexed with **PMMA-H** was found to be more stable after 90 days at 40°C with a lower decrease in antioxidant activity compared to the same formulation with free **QE**.

Instead, **QE** seemed to be generally more stable in the O/W cream because there was not a high decrease of antioxidant activity. In this formulation, in fact, the antioxidant activity of **QE** complexed with **PMMA-H** mantained the radical scavenger activity for 90 days while in the same formulation without **PMMA-H** decreased of 8.32%.

3.7 Qualitative characterization of drug-polymer complexes by Scanning Electron Microscopy

Drug-PMMA-H complexes that had been studied in terms of release by Franz cells are:

- ✓ **BZD-PMMA-H;**
- ✓ **IBUPROFEN-PMMA-H (loaded by Vaseline);**

- ✓ **IBUPROFEN-PMMAH-H (loaded by HMDS);**

- ✓ **GLA-PMMA-H.**

Each of these complexes, before being put inside topical formulations, in order to test the release, had been characterized by Scanning Electron Mycroscopy (SEM).
SEM had also been carried out on the blank polymer.

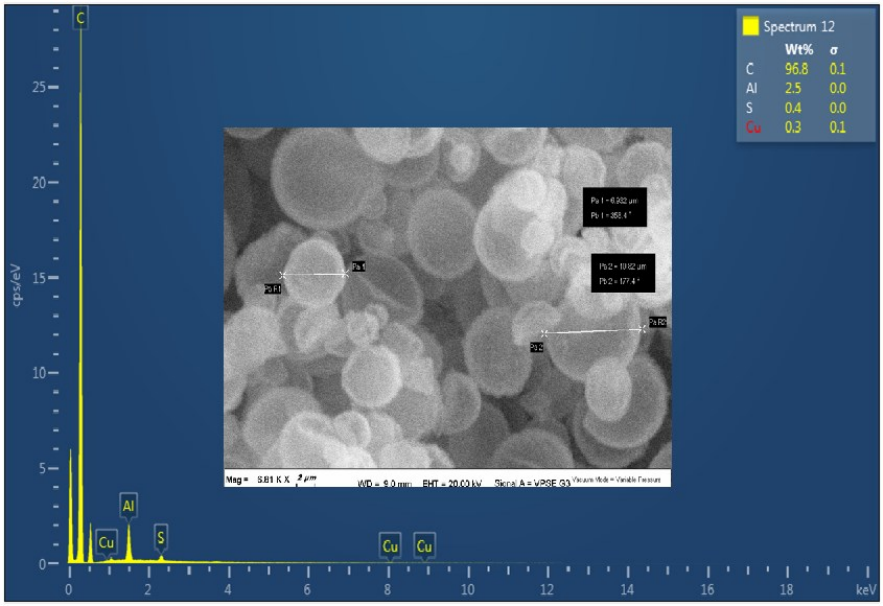


Figure 43. *SEM analisys of PMMA-H: Particle size, Chemical elements.*

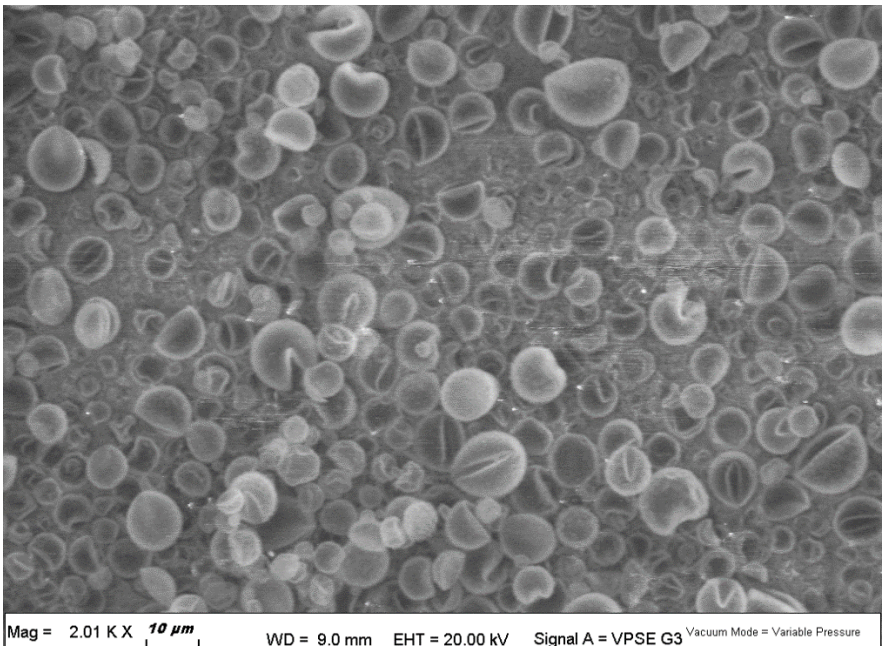


Figure 44. *SEM analisys of PMMA-H.*

SEM analysis of **PMMA-H** was shown in Figure 43 and 44. Empty polymer had little variability of particle size between 6 and 10 µm and same traces of impurity in very low percentage.

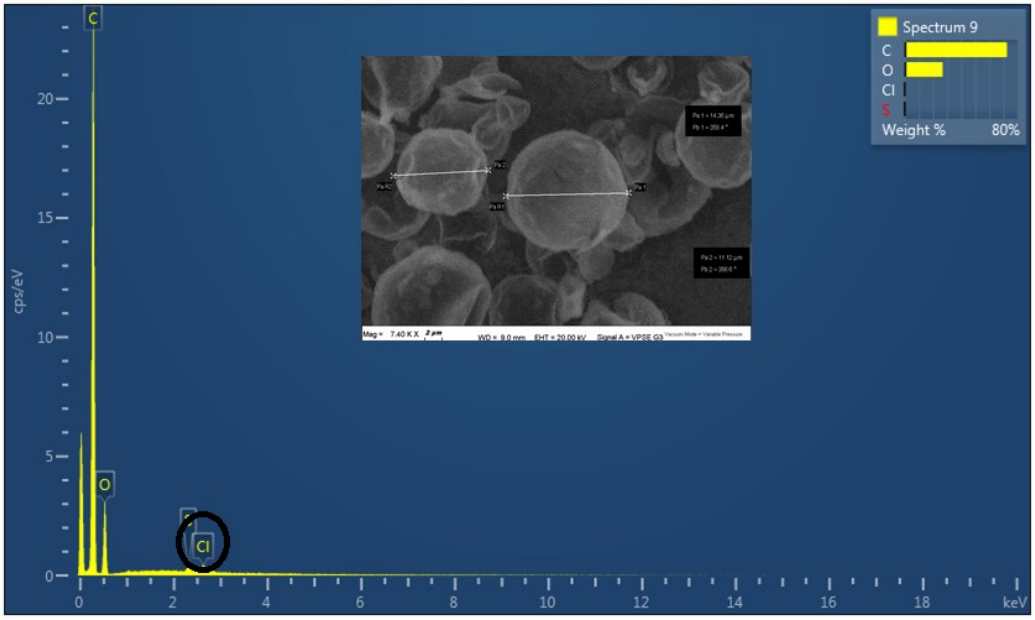


Figure 45. *SEM analisys of BZD-PMMA-H: Particle size, Chemical elements.*

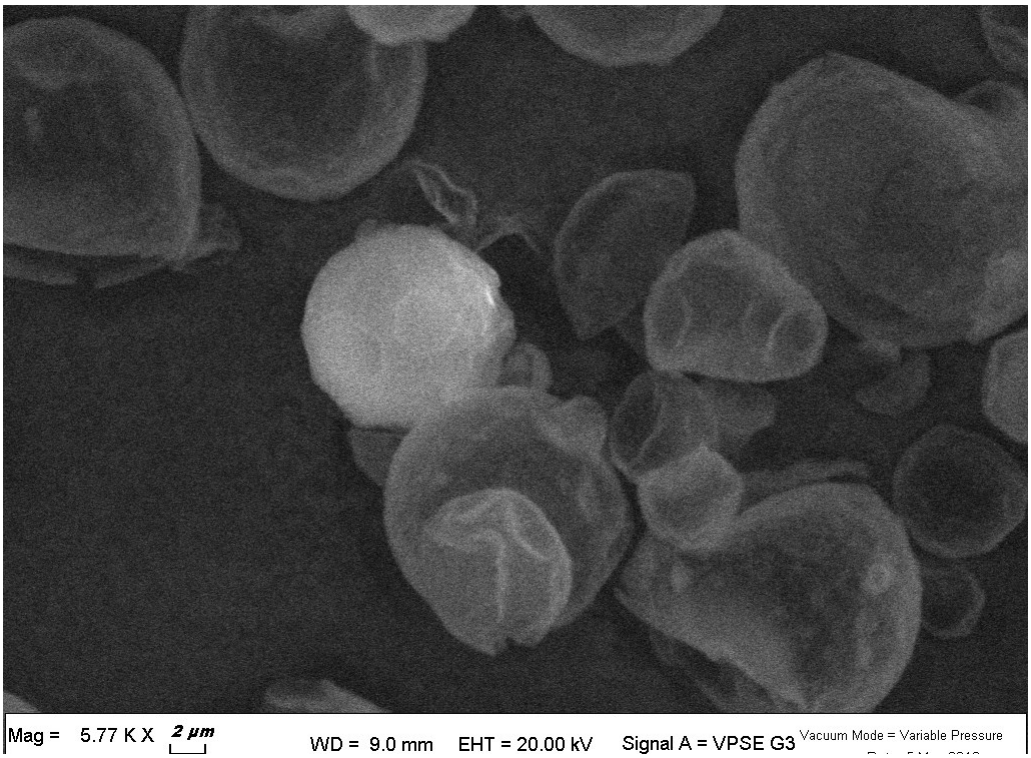


Figure 46. *SEM analisys of BZD-PMMA-H.*

Figures 45, and 46 show SEM analysis on **BZD-PMMA-H** complex: particle size resulted bigger than blank polymer (11-14 μm) and the analysis of the chemical elements show the presence of chlorine that confirmed the presence of Benzydamine hydrochloride. The outer surface of the polymer appeared to be unchanged, therefore the drug is into **PMMA-H** core.

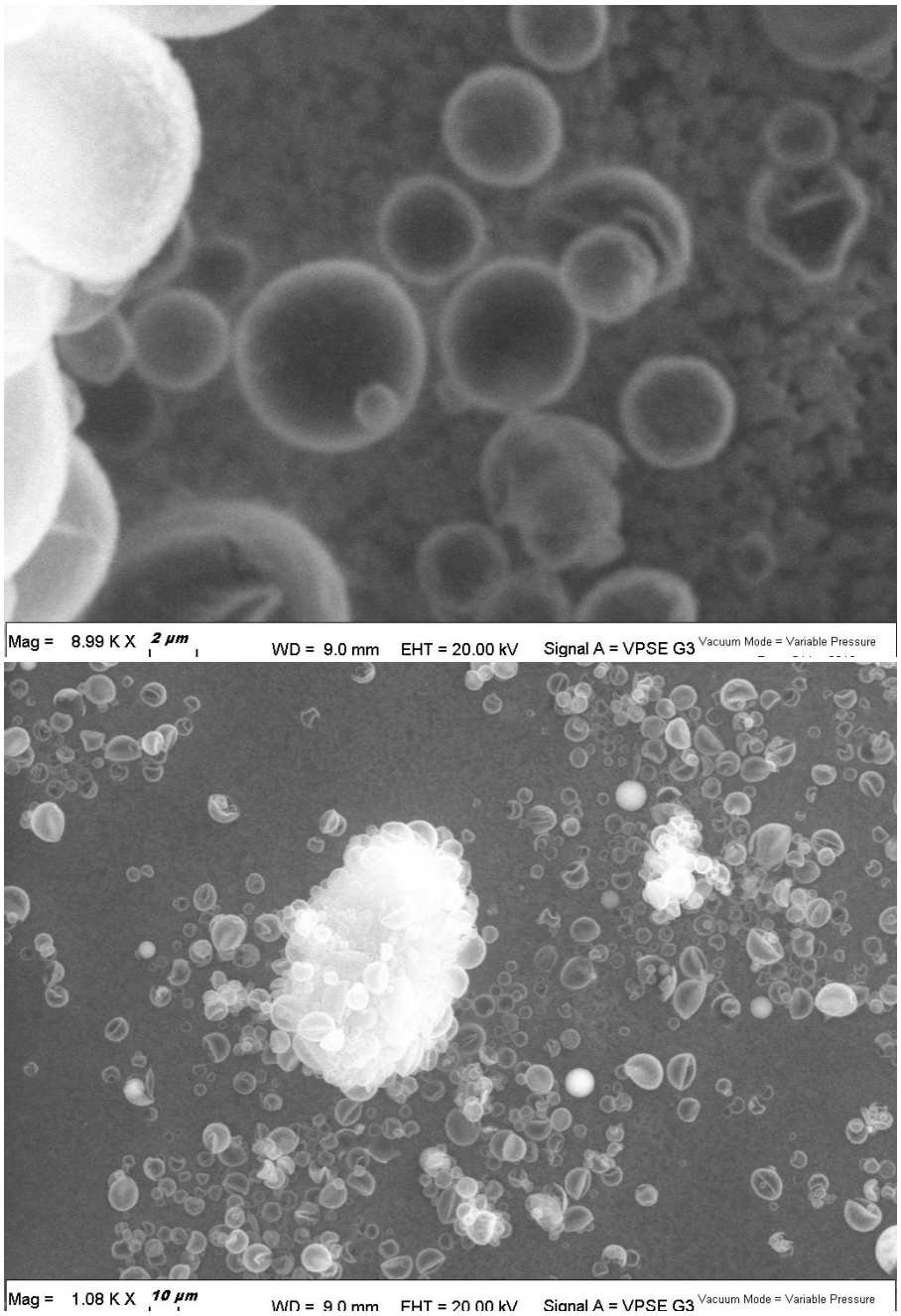


Figure 47. *SEM of **IBU-PMMA-H** loaded by Emds.*

Figure 47 shows SEM analysis of the **IBU-PMMA-H** complex (by HMDS): also in this case, the surface of the polymer appeared to be unchanged, there however were traces of solvent inside the particles.

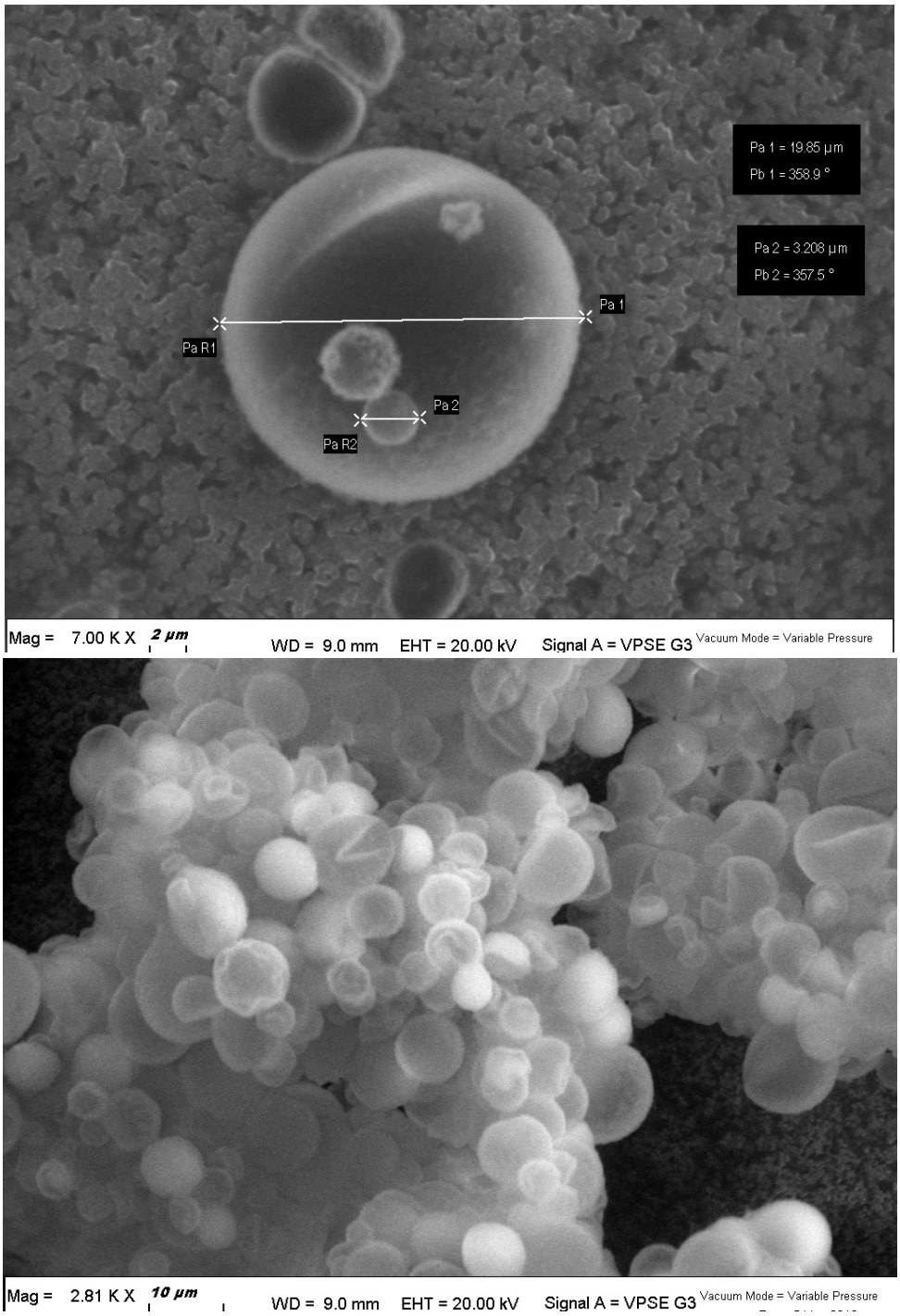


Figure 48. *SEM of **IBU-PMMA-H** loaded by Vaseline.*

Figure 48 shows SEM analysis of the **IBU-PMMA-H** complex (by Vaseline): also in this case the outer surface of the polymer appears to be unchanged, particle sizes were in the order of 20 µm and they are full of the oil phase.

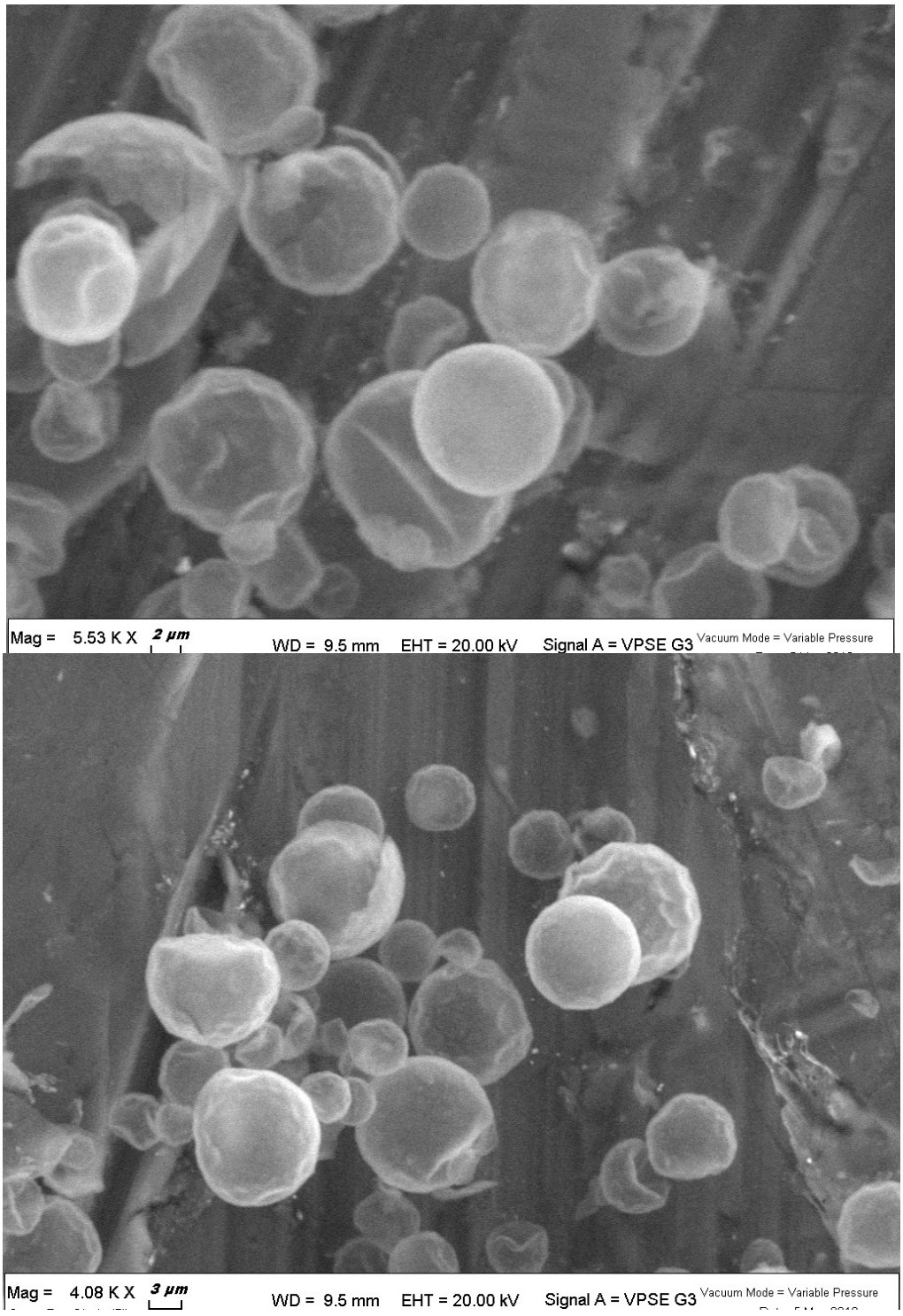


Figure 49. *SEM of* ***GLA-PMMA-H****.*

Figure 49 shows SEM analysis of **GLA-PMMA-H** complex. Particles were very similar to **BZD-PMMA-H**.

4. EXPERIMENTAL PART

4.1 Materials and methods

Reaction course was regularly monitored by thin-layer chromatography on pre-coated silica gel plates (Macherey-Nagel Durasil-25) by detection under a 254-nm UV lamp and using as eluent different mixtures of dichloromethane/methanol or butanol/ water/ acetic acid or petroleum ether/acetic acid.

^{1}H-NMR spectra were determinined in *d6*-DMSO and recorded÷ on VXR-200 Varian spectrometer and Mercury Plus-400. Chemical shifts are expressed in parts per million (ppm) using tetramethylsilane as the reference standard (TMS).

Reagents, solvents and standards samples were supplied by Sigma-Aldrich, Milan, Italy; Reagents Carlo Erba, Milan, Italy.

UV spectrophotometric analyses were carried out on a UV-VIS spectrophotometer (Shimadzu UV-2600) or on a Life Science UV/VIS *spectrophotometer* (*Beckman Coulter*™, DU®530, Single Cell Module)

HPLC analysis was performed using an Agilent 1100 Series HPLC System equipped with a G1315A DAD, autosampler and with a Phenomenex Synergi Hydro-RP C18 80Å column (4.6 × 150 mm, 4 μm).

4.2 Synthetic Procedures

4.2.1 Synthesis of Benzymidazolic compounds

- Synthesis of 2- (4-hydroxy-3-methoxyphenyl) -1H-benzo [d] imidazole-5-carboxylic acid (**AB101**)

In a round-bottomed flask (50 mL) to a solution of 3,4-diaminobenzoic acid (99.50 mg, 0.92 mmol) and 4-hydroxy-3-methoxybenzaldehyde (99.50 mg, 0.92 mmol) in methanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol). The reaction mixture was heated at 80°C under reflux for 17 hours. The solvent was then evaporated under reduced pressure. **AB101** was obtained after purification on column chromatography (Acetic Acid/MeOH 10:10) with ion exchange resin, (Dowex 1x2-400) (60% yield) as a beige powder.
^{1}H-NMR (DMSO) δ ppm: 3.90 (s, 3H); 6.95 (d, 1H); 7.62 (d, 2H); 7.80 (m, 2H); 8.18 (s, 1H); 10.85 (s, broad, 1H, -OH); 14-16 (s, broad, 2H, -COOH, -NH).ESI-MS: m/z found 285,32 Da $[M+H]^{+}$, m/z calculated for $C_{15}H_{12}N_2O_4$: 284,26. Mp. 133-135°C.

- Synthesis of INTERMEDIATE 1

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of 1-bromo-α-D-glucose tetraacetate ($2.06 \cdot 10^{3}$ mg, 2.50 mmol) in CH_2Cl_2 (5 mL) under argon were added benzyltriethylammonium chloride (114 mg, 0.50 mmol), potassium carbonate ($1.72 \cdot 10^{-3}$ mg, 12.50 mmol), and salicyl aldehyde (0.265 mL, 2.50 mmol). The reaction mixture was kept under magnetic stirring, at room temperature and in the dark for 24 h.
The mixture was neutralized with 8 mL of a solution of 10% HCl. The organic phase was washed first with 3x10 mL of a saturated solution of $NaHCO_3$ and with 3x10mL of a saturated solution of Brine. The organic phase obtained was then anhydrified and evaporated to dryness. Purification was effected by crystallization from MeOH to obtain **intermediate1** as a white powder.

^{1}H-NMR (DMSO) δ ppm: 2.02 (m, 12H); 4.15 (m, 1H); 4.22 (m, 1H); 4.32(m, 1H); 5.04 (t, 1H); 5.20 (m, 1H); 5.45 (m, 1H); 5.71(d, 1H); 7.25 (m, 2H); 7.73 (m, 2H); 10.17 (s, 1H, -CHO).ESI-MS: m/z found 453,18 Da $[M+H]^+$, m/z calculated for $C_{21}H_{24}O_{11}$: 452, 37.

- Synthesis of 2- (2-hydroxy-phenyl-o-β-D-glucose tetra acetate) -1H-benzo [d] imidazole (**AB102**)

In a round-bottomed flask (50 mL) a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol) was added to a solution of **intermediate 1** (416 mg, 0.92 mmol) and o-phenylenediamine (99.45 mg, 0.92 mmol) in methanol (5 mL).The reaction mixture was kept under magnetic stirring and was heated at 80°C under reflux for 3 h.

Then the reaction was evaporated to dryness under reduced pressure. The crude residue was dissolved in ethyl acetate and washed with water. The organic phase was dried, filtered and evaporated to give a crude residue purified by crystallization from ethyl ether to obtain **AB102** as a white powder (62.17% yield).

^{1}H-NMR (DMSO) δ ppm: 1.56 (s, 3H); 2.02 (m, 9H); 4.15 (m, 1H); 4.24 (m, 3H); 5.07(m, 1H); 5.45 (d, 2H); 5.78 (d, 1H); 7.25 (m, 4H); 7.53(m, 3H); 8.23 (m, 1H); 11.66 (s, 1H, -NH). ESI-MS: m/z found 541,71Da $[M+H]^+$, m/z calculated for $C_{27}H_{28}N_2O_{10}$: 540,49. Mp. 161-162.7°C.

- Synthesis of 2- (2-hydroxy-phenyl-o-β-D-glucose tetra acetate) -1H-benzo [d] imidazole-5-carboxylic acid (**AB103**)

In a round-bottomed flask (50 mL) to a solution of **intermediate 1** (416 mg, 0.92 mmol) and 3,4-diaminobenzoic acid (140 mg, 0.92 mmol) in methanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol).The reaction mixture was kept under magnetic stirring and was heated at 80°C under reflux for 2 h.

Then the reaction was evaporated to dryness under reduced pressure. The crude residue was dissolved in ethyl acetate and washed with water. The organic phase was dried, filtered and evaporated to give a crude residue purified by crystallization from methanol and petroleum ether to obtain **AB103** as a white powder (64.7% yield).

^{1}H-NMR (DMSO) δ ppm: 1.56 (s, 3H); 2.02 (m, 9H); 4.15 (m, 1H); 4.24 (m, 3H); 5.07(m, 1H); 5.45 (d, 2H); 5.78 (d, 1H); 7.25 (m, 4H); 7.53(m, 3H); 8.23 (m, 1H); 14-16 (s, broad, 2H, -COOH, -NH). ESI-MS: m/z found 585,7 Da $[M+H]^+$, m/z calculated for $C_{28}H_{28}N_2O_{12}$: 584,49. Mp. 174.9-175.3°C.

- Synthesis of INTERMEDIATE 2

In a round-bottomed flask (100 mL) to a solution of Helicin (397.20 mg, 1.40 mmol) and DMAP (733 mg, 6 mmol) in CH_2Cl_2 anhydrous (20 mL), was added propionyl chloride (0.96 mL, 6.56 mmol). The reaction mixture was stirred for 17 h under argon atmosphere. The reaction mixture was washed with H_2O and Brine. The organic phase was dried, filtered and evaporated to give a crude residue purified by chromatography over silica gel (CH_2Cl_2/MeOH 98:20) to obtain **intermediate 2** as a white powder.
^{1}H-NMR (DMSO) δ ppm: 1.01 (m, 12H); 2.29 (m, 8H); 4.16 (m, 3H); 5.16(m, 2H); 5.47 (t, 1H); 5.72 (m, 1H); 7.25 (m, 2H); 7.71(m, 2H); 7.25 (m, 2H); 10.17 (s, 1H, -CHO). ESI-MS: m/z found 509,72 Da $[M+H]^+$, m/z calculated for $C_{25}H_{32}O_{11}$:508,6.

- Synthesis of 2- (2-hydroxy-phenyl-o-β-D-glucose tetra propionate) -1H-benzo [d] imidazole (**AB104**)

In a round-bottomed flask (50 mL) to a solution of **intermediate 2** (467 mg, 0.92 mmol) and o-phenylenediamine (99.45 mg, 0.92 mmol) in methanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol).The reaction mixture was kept under magnetic stirring and was heated at 80°C under reflux for 2 h.
Then the reaction was evaporated to dryness under reduced pressure. The crude residue was dissolved in ethyl acetate and washed with water. The organic phase was dried, filtered and evaporated to give a crude residue purified by crystallization from ethyl ether to obtain **AB104** as a white powder (58.37% yield).
^{1}H-NMR (DMSO) δ ppm: 1.56 (m, 12H); 2.02 (m, 8H); 4.27 (m, 3H); 5.1 (m, 1H); 5.49 (m, 2H); 5.78 (d, 1H); 7.25 (m, 4H); 7.53(m, 3H); 8.23 (m, 1H); 11.64 (s, 1H, -NH). ESI-MS: m/z found 597,49 Da $[M+H]^+$, m/z calculated for $C_{31}H_{36}N_2O_{10}$: 596,63. Mp. 161-163°C.

4.2.2 Synthesis of Hesperidin's derivatives

Synthesis of ESP1

To a solution of Hesperidin (390.76 mg, 0.64 mmol) and DMAP ($2.81 \cdot 10^3$ mg, 23,04 mmol) in CH_2Cl_2 (40 mL), was added Tert-butyl chloride (1.9 mL, 17.28 mmol). After stirring at room temperature for 14 hours, the reaction mixture was washed with H_2O, $NaHCO_3$ and Brine. The organic phase was subsequently dried, filtered and evaporated under reduced pressure to give a crude residue purified by chromatography over silica gel (eluent: ethyl acetate) to obtain **ESP1** as orange oil (yield 65.25%).

^{1}H-NMR (DMSO) δ ppm: 0.96 (m, 69H); 2.09 (m, 12H); 2.43 (m, 2H); 2.50 (m, 2H); 2.70 (m, 1H); 3.30 (m, 2H); 3.60 (m, 1H); 3.80 (s, 3H); 4.30 (t, 1H); 5.10 (m, 4H); 5.43 (d, 1H); 5.55 (m, 1H); 5.80 (d, 1H); 6.40 (m, 2H); 7.20 (d, 2H), 7.40 (m, 1H); 12.00 (s, 1H). MW: 1297.56.

Synthesis of ESP2

To a solution of Hesperidin (390.76 mg, 0.64 mmol) and DMAP ($2.81 \cdot 10^3$ mg, 23.04 mmol) in CH_2Cl_2 (40 mL), Cyclohexyl-acetyl chloride was added (2.64 mL, 17.28 mmol). After stirring at room temperature for 14 hours, the reaction mixture was washed with H_2O, $NaHCO_3$ and Brine. The organic phase was subsequently dried, filtered and evaporated under reduced pressure to give a crude residue purified by chromatography over silica gel (eluent: ethyl acetate) to obtain **ESP2** (final yield 79.28%) as orange oil.

^{1}H-NMR (DMSO) δ ppm: 1.20 (t, 3H); 1.38 (m, 36H); 1.80 (m, 24H); 2.09 (m, 12H); 2.43 (s, 2H); 2.50 (m, 2H); 2.70 (m, 1H); 3.30 (m, 2H); 3. 60 (m, 1H); 3.80 (s, 3H); 4.30 (t, 1H); 5.10 (m, 4H); 5.43 (d, 1H); 5.55 (m, 1H); 5.80 (d, 1H); 6.45 (m, 2H); 7.20 (d, 2H), 7.40 (m, 1H); 9.00 (s, 1H); 12.00 (s, 1H).

MW: 1355.64.

Synthesis of ESP3

To a solution of Hesperidin (390.76 mg, 0.64 mmol) and DMAP ($2.81 \cdot 10^3$ mg, 23.04 mmol) in CH_2Cl_2 (40 mL), benzoyl chloride was added (2.01 mL, 17.28 mmol). After stirring at room temperature for 14 hours, the reaction mixture was washed with H_2O, $NaHCO_3$ and Brine. The organic phase was subsequently dried, filtered and evaporated under reduced pressure to give a crude residue purified by chromatography over silica gel (eluent: ethyl acetate). **ESP2** was obtain by crystallization from methanol as a light yellow powder (final yield 56.87%).

^{1}H-NMR (DMSO) δ ppm: 1.20 (t, 3H); 2.43 (s, 2H); 2.70 (m, 1H); 3.30 (m, 2H); 3.60 (m, 1H); 3.80 (s, 3H); 4.30 (t, 1H); 5.10 (m, 4H); 5.43 (d, 1H); 5.55 (m, 1H); 5.80 (d, 1H); 6.45 (m, 2H); 7.20 (d, 2H), 7.40 (m, 1H); 7.52 (m, 14H); 7.62 (m, 7H); 7.97 (m, 14H); 12.00 (s, 1H). MW: 1339.30.

Synthesis of ESP4

To a solution of Hesperidin (390.76 mg, 0.64 mmol) and DMAP ($2.81 \cdot 10^3$ mg, 23.04 mmol) in CH_2Cl_2 (40 mL), was added Eptanoil chloride ($7.57 \cdot 10^{-3}$ mg, 17.28 mmol). After stirring at room temperature for 14 hours, the reaction mixture was washed with H_2O, $NaHCO_3$ and Brine. The organic phase was subsequently dried, filtered and evaporated under reduced pressure to give a crude residue purified by chromatography over silica gel (eluent: petroleum ether/ethyl acetate: 80/20). **ESP4** was obtain as orange oil (final yield 27.70%).

^{1}H-NMR (DMSO) δ ppm: 0.95 (m, 18H); 1.20 (t, 3H); 1.28 (m, 24H); 1.33 (m, 12H); 1.68 (m, 12H); 2.20 (m, 12H); 2.43 (s, 2H); 2.70 (m, 1H); 3.30 (m, 2H); 3.60 (m, 1H); 3.80 (s, 3H); 4.30 (t, 1H); 5.10 (m, 4H); 5.43 (d, 1H); 5.55 (m, 1H); 5.80 (d, 1H); 6.45 (m, 2H); 7.20 (d, 2H), 7.40 (m, 1H); 9.00 (s, 1H); 12.00 (s, 1H). MW: 1283.58.

Synthesis of ESP5

To a solution of Hesperidin (390.76 mg, 0.64 mmol) and DMAP ($2.81 \cdot 10^3$ mg, 23.04 mmol) in CH_2Cl_2 (40 mL), was added propionyl chloride (1.45 mL, 17.28 mmol).

After stirring at room temperature for 14 hours, the reaction mixture was washed with H_2O, $NaHCO_3$ and Brine. The organic phase was subsequently dried, filtered and evaporated under reduced pressure to give a crude residue purified by chromatography over silica gel (eluent: ethyl acetate). **ESP5** was obtain as yellow powder (final yield 33.60%).

^{1}H-NMR (DMSO) δ ppm: 1.15 (m, 21H); 2.28 (m, 14H); 2.43 (s, 2H); 2.70 (m, 1H); 3.30 (m, 2H); 3.60 (m, 1H); 3.80 (s, 3H); 4.30 (t, 1H); 5.10 (m, 4H); 5.43 (d, 1H); 5.55 (m, 1H); 5.80 (d, 1H); 6.45 (m, 2H); 7.20 (d, 2H), 7.40 (m, 1H); 12.00 (s, 1H). M.W: 1003.00.

Synthesis of ESP6

To a solution of Hesperidin (390.76 mg, 0.64 mmol) and DMAP ($2.81 \cdot 10^3$ mg, 23.04 mmol) in CH_2Cl_2 (40 mL), was added naphthoyl chloride ($3.29 \cdot 10^3$ mg, 17.28 mmol). After stirring at room temperature for 14 hours, the reaction mixture was washed with H_2O, $NaHCO_3$ and Brine. The organic phase was subsequently dried, filtered and evaporated under reduced pressure to give a crude residue purified by chromatography over silica gel (eluent: petroleum ether/ethyl acetate: 50/50). **ESP6** was obtain as white powder (final yield 77.23%).

^{1}H-NMR (DMSO) δ ppm: 1.20 (t, 3H); 2.43 (s, 2H); 2.70 (m, 1H); 3.30 (m, 2H); 3.60 (m, 1H); 3.80 (s, 3H); 4.30 (t, 1H); 5.10 (m, 4H); 5.43 (d, 1H); 5.55 (m, 1H); 5.80 (d, 1H); 6.45 (m, 2H); 7.20 (d, 2H), 7.40 (m, 1H); 7.37 (m, 35H); 8.08 (m, 14H); 12.00 (s, 1H). M.W: 1689.71

Synthesis of ESP7

To a solution of Hesperidin (390.76 mg, 0.64 mmol) and DMAP ($2.81 \cdot 10^3$ mg, 23.04 mmol) in CH_2Cl_2 (40 mL), was added cinnamoyl chloride ($2.88 \cdot 10^3$ mg, 17.28 mmol). After stirring at room temperature for 14 hours, the reaction mixture was washed with H_2O, $NaHCO_3$ and Brine. The organic phase was subsequently dried, filtered and evaporated under reduced pressure to give a crude residue purified by

chromatography over silica gel (eluent: petroleum ether/ethyl acetate: 52/48). **ESP7** was obtain as light yellow powder (final yield 74.07%).

^{1}H-NMR (DMSO) δ ppm: 1.20 (t, 3H); 2.43 (s, 2H); 2.70 (m, 1H); 3.30 (m, 2H); 3.60 (m, 1H); 3.80 (s, 3H); 4.30 (t, 1H); 5.10 (m, 4H); 5.43 (d, 1H); 5.55 (m, 1H); 5.80 (d, 1H); 6.45 (m, 2H); 6.80 (d, 8H); 7.20 (d, 2H), 7.40 (m, 1H); 7.53 (m, 16H); 7.63 (m, 8H); 7.74 (d, 8H); 7.98 (m, 16H). M.W: 1705.80.

4.2.3 Synthesis of Baicalin's derivative

In a round-bottomed flask (50 mL) to a solution of Baicalin (446 mg, 1 mmol) in Acetic anhydride (4 mL) was added sodium acetate (138.24 mg, 1.665 mmol). The reaction mixture was kept under magnetic stirring and was heated at 75°C under reflux for 2 hours and half.

Then to the reaction was added few milliliters of water to obtain the crude residue as crystals. Crystals were filtered under vacuum and washed with ethanol to obtain the desired product **B1** as a white powder color dirt (final yield 71%).

^{1}H-NMR (DMSO) δ ppm: 2.21 (m, 15H); 4.75 (m, 5H); 7.14 (s,1H); 7.24 (s,1H); 7.60 (d, 3H); 8.12 (d, 2H); 12.89 (s, 1H). M.W: 656.

4.2.4 Synthesis of Salicin's derivative

In a round-bottomed flask (50 mL) to a solution of Salicin (143.14 mg, 0.5 mmol) in Acetic anhydride (2 mL) was added sodium acetate (69.12 mg, 0.83 mmol). The reaction mixture was kept under magnetic stirring and was heated at 75°C under reflux for about 3 hours.

Then to the reaction was added few milliliters of water to obtain the crude residue as crystals. Crystals were filtered under vacuum and washed with water to obtain the desired product **SA1** as a white powder (final yield 62%).

^{1}H-NMR (DMSO) δ ppm: 2.01 (m, 15H); 4.20 (m, 3H); 4.96 (m, 2H); 5.05 (m, 2H); 5.43 (m, 2H); 7.10 (m, 2H); 7.32 (m, 2H). M.W: 496.28.

4.3 Description of methods used for the evaluation of the biological activity

4.3.1 Antioxidant activities

4.3.1.a ORAC test

The ORAC assay was carried out on a Fluoroskan FL® ascent (Thermo Fisher Scientific, Inc. Waltham, MA, USA) with fluorescent filters. The procedure was based on that given by Hong, Guohua & Ronald [95.96].

Samples, Trolox standards and all other reagents were prepared at 75 mM phosphate buffer (PBS) (pH 7.4-7.44). Briefly, in the final assay mixture (0.2 mL total volume), fluorescein sodium salt (85 nM) was used as a target of free radical attack with 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator. PBS was used as blank. Trolox®, used as control standard, was dissolved in 5 mL of PBS and suitably diluted to the following concentrations: 2, 4, 6, 8, 10 nanomolar.

Instrument was programmed to record kinetically the fluorescence of fluorescein after the addition of AAPH . All measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the fluorescence decay curves between the blank and a sample. Results were expressed as micromoles Trolox equivalent (TE).

4.3.1.b DPPH Test

To 1.5 mL DPPH methanolic solution (0.5 mM) was added 0.750 mL of sample solution proper diluted. Samples absorbance measurements were evaluated with a Life Science UV-VIS spectrophotometer (*Beckman Coulter*™, DU®530, Single Cell Module) at fixed wavelength of 517 nm. Blank sample was prepared adding methanol to DPPH solution and Trolox was used as standard reference to achieve a calibration curve. The radical-scavenging activity is expressed as inhibition ratio of initial concentration of DPPH radical and is calculated according to the formula:

Inhibition percentage (Ip) = [(AB-As)/AB] · 100; where AB and As are, respectively, the absorbance values of blank reaction and of the tested sample 94.

4.3.1.c FRAP Test

The reagent for analysis was freshly prepared by mixing the following solutions in the reported ratio 10/1/1 (v:v:v):

i) 0.1 M acetate buffer pH 3.6,

ii) TPTZ 10 mmol/L in 40 mmol/ HCl,

iii) ferric chloride 20 mmol/L.

To a 1.9 mL of reagent were added 0.1 mL of sample proper diluted or solvent when blank was performed. Readings at fixed wavelength of the absorption maximum (593 nm) were done after 30 min, using a UV-VIS spectrophotometer; it was evaluated the absorbance increase of sample solution against the absorbance of blank reaction as parameter to calculate the antioxidant activity. The antioxidant activity is given as Trolox activity since this standard was used to perform the calibration curves.

4.3.1.d PCL Test

Photochemiluminescence (PCL) assay was performered by means of Photochem® apparatus using PCL Kits purchased by Analytik-Jena AG (Jena, Germany). Production of superoxide anion radicals is sensitized from luminol after exposure to UV light lamp (Double Bore® phosphorus lamp, output 351 nm, 3 mWatt/cm^2). The antioxidant activity was measured using ACL method (Antioxidant Capacity of Liposoluble).

To a volume included between 5 and 25 μL of of standard Trolox solution 0.1mM (to obtain the calibration curve) or sample solution proper diluted were added and mixed a 2.30 mL portion of reagent 1 (HPLC-grade methanol), 0.2 mL of reagent 2 (buffer solution), 25 μL of reagent 3 (photosensitizer). The mixture solution was then measured, during a time of 130 seconds, by means of Photochem®. The antioxidant

capacity of the sample is calculated by comparison with a Trolox standard curve. The areas under the reaction curves were calculated using the PCLsoft control and analysis software; at greater concentrations of Trolox working solutions correspond a marked reduction in the magnitude of the PCL signal and hence a reduction of the area under the curve. This inhibition was used as a parameter for quantification of antioxidant capacity.

4.3.2 Antifungal activity

4.3.2.a Microrganisms

The dermatophytes used were *Arthroderma Cajetani Ajello*, CBS 495.70 strain; *Epidermophyton floccosum* (Hartz) Langerone Milochevitch, CBS 358.93 strain; *Trichophyton violaceum* Malmsten, CBS 459.61 strain; *Trichophyton tonsurans* Malmsten, CBS483.76 strain, *Trichophyton mentagrophytes* (Robin) Blanchard, CBS 160.66 strain; *Microsporum canis* Bodin CBS 4727 strain; *Nannizzia Gypsea* (Bodin) Guiart et Grigoraki CBS 286.63 strain purchased by the Centraal Bureau voor Schimmelcultures (CBS), Baarn, Netherlands; *Trichophyton rubrum* (Castellani) Sabouraud IHME 4321; *Microsporum gypseum* (Bodin) Guiarte Grigorakis IHME 3999 from Institute of Hygiene and Epidemiology-Mycology (IHME, Brussels, Belgium).

Cultures were maintained at 4°C as agar slants, on Sabouraud Dextrose Agar (SDA, Sigma-Aldrich SRL, Milan, Italy).

4.3.2.b Growth inhibition

Antifungal activity was determined as follows. Each test substance was dissolved in dimethyl sulfoxide (DMSO), and a suitable dilution was aseptically mixed with sterile SDA medium at 45 °C to obtain final concentrations of 20 and 100 μg/mL.

The DMSO concentration in the final solution was adjusted to 0.1%. Controls were also prepared with equivalent concentrations (0.1% v/v) of DMSO. For the experiments, cultures were obtained by transplanting mycelium disks (10 mm in diameter) from a single mother culture in the stationary phase. They were incubated at 26 ± 1 °C on SDA on thin sheets of cellophane until the logarithmic growth phase. Subsequently, the cultures were transferred to Petri dishes with media containing 20 and 100 μg/mL of the tested substances and incubated under growth conditions. The fungal growth was evaluated daily by measuring the colony diameters (in millimetres) for seven days beginning at the onset of treatment. The percentage inhibition of growth was determined as the average of three different experiments.
The percentage of inhibition compared to control is calculated according to the following equation:

$$\text{Percentage of relative inhibition (\%)} = [(dex - dex') / dex] \times 100\%$$

Where dex is the diameter of the mycelium in the control; and dex' is the diameter of the mycelium measured in the experiment plates at different concentrations.

4.3.3 Cosmetic Formulations and Evaluation of Filtering Parameters

The new synthesized compounds (**AB101**, **AB102**, **AB103** and **AB104**) were included at the concentration of 1% in a cosmetic formulation containing AVB (3%) and OMC (6%) to demonstrate the effective filtering capacity of finished formulation.

INCI: Aqua, Tribehenin PEG-20 esters, Octyl metoxycinnamate, Glycerin, Avobenzone, Cetearyl alcohol, Phenoxyethanol, Propylene glycol, Methylparaben, Ethylparaben, Propylparaben, Butylparaben, Polydimethylsiloxane.

The substrates chosen for the analysis were PMMA (polymethylmethacrylate) plates, type WW2 standardized for this use and provided in ISO 24443 for the spectrophotometric determination of UVAPF. Transmittance and absorbance measurements were carried out by a Shimadzu UV-2600 model equipped with integrating sphere ISR 2600 60 mm coupled with a SPF determination software. Spectra are recorded in the range 290-400nm.

The cream samples were prepared as follows: on the plate of Poly methyl methacrylate 0.0320 g of the product and this was then placed on a digital scale. Wearing a glove finger extends the product by applying the desired pressure (+/- 15g) by running six horizontal and vertical circular motion.

The plate was placed in a dark room for 15 minutes and then it was inserted into the instrument for measurement. Five measurements were made by turning the plate to each. The test was repeated three times. The blank was prepared using the HD6 plates covered with 15µL of glycerin, because of its non-fluorescence and UV transparency.
$SPF_{in\ vitro}$ was calculated using Equation 4:

Equation 4. *Calculation of in vitro SPF value.*

E(λ) is spectral irradiance of terrestrial sunlight that represents the midday midsummer sunlight for Southern Europe (latitude 40° Nord, solar zenith angle 20°, ozone layer 0,305 cm); B(λ) is the relative effectiveness of UVR at wavelength λ (nm) in producing delayed erythema in human skin.
UVAPF0 was calculated using Equation 5:

$$UVAPF0 = \int_{\lambda=320nm}^{\lambda=400nm} P(\lambda) \times I(\lambda) \times d\lambda$$

$$\int_{\lambda=290nm}^{\lambda=400nm} R(\lambda) \times I(\lambda) \times 10^{-A0(\lambda) \times C \times d\lambda}$$

$$SPF = \frac{\sum_{290}^{400} E(\lambda) B(\lambda)}{\sum_{290}^{400} E(\lambda) B(\lambda) T(\lambda)}$$

Equation 5. *Calculation of UVAPF0.*

4.4 Loading methods

4.4.1 Salicin-PMMA-H/PMMA-PH/PMMA-P complex

To a **PMMA-H/ PMMA-PH/ PMMA-P** solution (3.00g) in acetone (50mL), was added Salicin (0.750g) and Sucrose Stearate (0.43g). This system was left under stirring for 20 minutes at 10°C, and then it was emulsified by Turbo with liquid Vaseline (200 mL), also previously cooled to 10°C. The emulsion that was formed, was left under stirring for 24h, in order to allow evaporation of the organic solvent. The complex was filtered under vacuum, washing with hexane and dried at 50°C.

SALICIN-PMMA-H:

^{1}H-NMR (DMSO) δ ppm: 1.23 (s, 2H); 3.52 (s, 3H); 4.20 (m,3H); 4.96 (m, 2H); 5.05 (m,2H); 5.43 (m,2H); 7.10 (m, 2H); 7.32 (m, 2H).

SALICIN-PMMA-PH:

^{1}H-NMR (DMSO) δ ppm: 1.23 (s, 2H); 3.52 (s, 3H); 4.20 (m,3H); 4.96 (m, 2H); 5.05 (m,2H); 5.43 (m,2H); 7.10 (m, 2H); 7.32 (m, 2H).

SALICIN-PMMA-P:

^{1}H-NMR (DMSO) δ ppm: 1.23 (s, 2H); 3.52 (s, 3H); 4.20 (m, 3H); 4.96 (m, 2H); 5.05 (m, 2H); 5.43 (m, 2H); 7.10 (m, 2H); 7.32 (m, 2H).

4.4.2 Baicalin-PMMA-H complex

To a suspension of **PMMA-H** (3.20g) in acetone (50mL), Baicalin (800 mg, 1.79mmol) and Sucrose Stearate (460 mg, 0.75 mmol) were added, this system was left under stirring for 20 minutes at 10°C, then it emulsified by Turbo with liquid

Vaseline (200 mL), also previously cooled to 10°C. The emulsion that was formed, was left under stirring for 24h, in order to allow evaporation of the organic solvent. The complex was filtered under vacuum, washing with hexane and dried at 50°C.

^{1}H-NMR (DMSO) δ ppm: 1.23 (s, 2H); 3.52 (s, 3H); 4.75 (m, 5H); 7.14 (s, 1H); 7.24 (s, 1H); 7.60 (d, 3H); 8.12 (d, 2H); 12.89 (s, 1H).

4.4.3 Loading method by Ethanol

10% loading: The active ingredient (1.00 g) dissolved in EtOH (80 mL) was added very slowly to a **PMMA-H** suspension (9.00g) in EtOH (80mL). The new suspension was left under stirring for about an hour. The solvent was dried by rotavapor.

Yield obtained:

BZD-PMMA-H 10%: 98.00%

20% loading: The active ingredient (2.00g) dissolved in EtOH (40mL) was added very slowly to a **PMMA-H** suspension (8.00g) in EtOH (60mL). The new suspension was left under stirring for about an hour. Then, organic solvent was dried using rotavapor.

Yields obtained:

BZD-PMMA-H 20%: 97.50%

GLA-PMMA-H 20%: 98.87%

QE-PMMA-H 20%: 99.51%

4.4.4 Loading method by Oil

10% loading: To a solution of active ingredient (0.50g) dissolved in oil (Vaseline or HMDS) (10mL), **PMMA-H** (4.50g) was added very slowly. Complex was left under stirring until the polymer absorbed all the oil phase.

Yields obtained:

IBU-PMMA-H Vaseline 10%: 99.10%

IBU-PMMA-H HMDS 10%: 97.20%

20% loading: To a solution of an active ingredient (1.00g) in oil (Vaseline or HMDS) (10mL), **PMMA-H** (4.00g) was added very slowly. Complex was left under stirring until the polymer absorbed all the oil phase.

Yields obtained:

IBU-PMMA-H Vaseline 20%: 99.60%

IBU-PMMA-H EMDS 20%: 98.60%

4.5 Percentage of loading and release study

4.5.1 Concentration of drug in the complex

Each sample of the complex obtained was analyzed by UV spectroscopy in the range of (400-200 nm) to assess the percentage of active ingredient present within each complex. The concentration was calculated by interpolation from the calibration curve, using the absorbance values of samples of each complex. Each complex was weighed in the range of 50-100 mg and filtered before the analysis.

The extraction solvent was selected for each drug.

λ_{MAX} Salicin: 273 nm

λ_{MAX} Baicalin: 280 nm

λ_{MAX} BZD: 306 nm

λ_{MAX} Ibuprofen: 222 nm

λ_{MAX} GLA: 258 nm

λ_{MAX} QE: 256 nm

4.5.2 Dissolution test

The dissolution medium, phosphate buffer (400 mL) pH 7.4 (for Salicyn, Baicalin, SA1, B1 and IBU) and H_2O for BZD, was maintained at 37 ± 1°C by using a thermostated bath. Samples (2 mL) were taken from dissolution medium and tested by UV.

For each sample, the volume was replaced with new dissolution medium.

Each sample was filtered by 0.45μm filters before testing. The concentration was calculated by interpolation from the calibration curve.

4.5.3 In vitro release

4.5.3.a Formulations

Gel: Aqua, Glycerin, Hydroxyethylcellulose.

Lipogel: Caprylic/Capric Triglyceride, Propylene Glycol, Polyethylene glycol 400, Menthol.

Cream: Aqua, PEG-6 Stearate, Ethylene glycol stearate, PEG-32 stearate, Caprylic/Capric Triglyceride

O/W Emulsion: Aqua, Polyglyceryl-6 distearate, Jojoba esters, Polyglyceryl-3-beeswax, Cetyl alcohol, Caprylic/Capric Triglyceride, Prunus Amygdalus Dulcis Oil, Coco caprylate, Butyrospermum Parkii, Propanediol, Xantan gum, Carbomer, Hexanediol, Caprylyl glycol, Sodium acrylates copolymer/Hydrogenated polyisobutene, Phospholipids, Polyglyceryl-10 stearate, Helianthus annuus seed oil, Quercetin, EDTE Poloxamer 338, PPG-12/SMDI Copolymer Fenoxyethanol, Triethanolamine.

W/O Emulsion: Aqua, Caprylic/Capric Triglyceride, Isoamyl cocoate, Simmondsia

Chinensis seed oil, Glycerin, Polyglyceryl-4-diisostearate/Polyhydroxystearate/Sebacate, Hydrogenated castor oil, Cera Alba, Panthenol, Magnesium Sulfate Heptahydrate, Fenoxyethanol, Quercetin, Citric acid.

4.5.3.b Franz cell system

Franz cell method parameters:

- Surface: 0.6 cm^2
- Membrane: regenerated cellulose (45μm)
- Donor phase: dose infinita (1.00g circa)
- Receptor phase: different solvent according to the solubility
- Stirring speed: 100 rpm
- Sampling: 0.4 mL at every time point, replaced with fresh receptor solvent
- Sample analysis: direct UV analysis at λ_{MAX}

Membrane was conditioned with the receiver solution (24h early) before being fitted to the diffusion cell. For each formulation, the best solvent, that was able to maintain sink condition during release experiments, was studied. The samples were taken every hour for eight hours and analyzed by UV. The solvent chosen for each molecule is reported in Table 16.

Drug	Solvent
BZD	H_2O
IBU	Phosphate Buffer p.H. 7.4
GLA	Phosphate Buffer p.H. 11.5

Table 16. *Solvent chosen for Franc cell test.*

4.6 Stability studies

4.6.1 Stability studies on Hesperidin and derivatives

In this study, Hesperidin and semisynthetic derivatives **Esp1** - **Esp7** had been incorporated in cosmetic formulations, then subjected to accelerated aging in a stove at 40°C and tested by HPLC with the aim of assessing their contents. The study was conducted on each formulation containing 0.3% of the active ingredient.

The tested formulation was prepared as follows:

INCI: aqua, *Tribehenin PEG-20 Esters,* Caprylic/Capric Triglyceride, *Dicaprylyl Ether,* glycerin, cetyl stearyl alcohol, Phenoxyethanol, Propylene glycol, Methylparaben, Propylparaben, Ethylparaben, Butylparaben, dimethicone.

From a technological point of view, it was a cosmetic O/W emulsion.

All formulations have been subjected to accelerated aging in a stove at 40°C and then analyzed by HPLC with the aim of evaluating the content of the active ingredient during 6 months.

Different mobile phase was chosen for each formulation:

(1) **Hesperidin**: The determination was performed in isocratic (77% H_2O / 23% CH_3CN). The separation was monitored by detecting the absorbance at 280 nm ± 8. The flow of the mobile phase was 1.2 mL / min, the injection volume of 5 µL and the separation was performed with thermostated column at 27°C. The analysis time was 9 minutes and the retention time of Hesperidin was t_R = 5.1 minutes.

(2) **ESP 1**: The determination was performed in isocratic (2% H_2O / 98% MeOH). The separation was monitored by detecting the absorbance at 270 nm ± 8. The flow of the mobile phase was 1.5 mL / min, the injection volume of 5 µL e the

separation was performed with thermostated column at 27 ° C. The analysis time was 13 minutes and the retention time was tR = 6.35 minutes.

(3) **ESP2**: The determination was performed in isocratic (77% H_2O / 23% CH_3CN). The separation was monitored by detecting the absorbance at 280 nm ± 8. The flow of the mobile phase was 1.2 mL / min, the injection volume of 5 µL, the separation was performed with thermostated column at 27 ° C. The analysis time was 9 minutes and the retention time was tR = 5.1 minutes.

(4) **ESP3**: The determination was performed in isocratic (5% H_2O / 95% CH_3CN). The separation was monitored by detecting the absorbance at 280 nm ± 8. The flow of the mobile phase was 1.2 mL / min, the injection volume of 5 µL, the separation was performed with thermostated column at 27 ° C. The analysis time was 11 minutes and the retention time was tR = 4.12 minutes.

(5) **ESP6**: The determination was performed in isocratic (55% H_2O / 45% CH_3CN). The separation was monitored by detecting the absorbance at 280 nm ± 8. The flow of the mobile phase was 1.2 mL / min, the injection volume of 5 µL, the separation was performed with thermostated column at 27 ° C. The analysis time was 11 minutes and the retention time was tR = 4.72 minutes.

(6) **ESP7**: The determination was performed in isocratic (5% H_2O / 95% CH_3CN). The separation was monitored by detecting the absorbance at 280 nm ± 8. The flow of the mobile phase was 1.2 mL / min, the injection volume of 5 µL, the separation was performed with thermostated column at 27 ° C. The analysis time was 13 minutes and the retention time was tR = 7.22 minutes.

5.0 CONCLUSIONS

5.1 Benzymidazolic compounds

Four molecules whith benzimidazolic ring were synthesized with the intent to obtain compounds with dualistic activity: antioxidant and UV filter.

All of the synthetic procedures allowed us to obtain the expected products with good yields, up to 50% for each compounds except for **AB104** that was synthetized with a yield around 30% (anyway a good result).

These compounds were *in vitro* tested to assess their biological properties: we have investigated antifungal activity, antioxidant and UV-filter capacity.

None of the four molecules showed interesting antifungal activity: only **AB104**, although in low percentages, was able to inhibt the growth of all eight dermatophytes used for the analysis, the other compounds (**AB101**, **AB102** and **AB103**) had increased the growth.

AB102 and **AB104** were dissimilar each other only by the presence of a methylene group in protection of the hydroxyl groups on the sugar portion of the molecule (made with acetate and propionate groups respectively): this change was sufficient to modify their biological activity.

As expected, compound **AB101** showed a good antioxidant activity against superoxide anion (PCL test), due to the presence of a free phenolic functionality, while, the other compounds, which this functionality was protected by a glycosidic bond, showed no antioxidant activity. However, we can assume that an *in vivo* test could show similar antioxidant activity of all four compounds, thanks to the presence in the organism of enzymes capable of splitting the glycosidic bond and liberate the phenolic functionality protected.

The critical λ values showed that none of all compounds can be considered a broad-spectrum sunscreen (all absorbtions are between 340 and 323 nm) but they were perfectly in the range of UVB radiation.

The synthesized molecules have been incorporated in cosmetic formulations with known sunscreens to evaluate a possible synergistic effect. In the case of **AB102** and

AB104, critical λ and SPF had significantly changed compared to the formulation in which they were absent, indeed, the compound **AB101** has a negative effect, with the lowering of the SPF value of a unit.

The best result was obtained with **AB103** that produced an increase of about 5 units in the SPF value, probably due to the fact that the carboxylic group may have a bathochromic effect on the molecule. It therefore seems possible to increase this result by modulating its amount in the formulation.

5.2. Hesperidin derivatives

Hesperidin is a natural flavonoid present in the *Citrus* plant. Previously studies carried out on its properties have confirmed, in particular, its antioxidant activity, anti-inflammatory, anti-cancer as well as other numerous beneficial properties for humans.

Being a polyphenolic compound, Hesperidin is characterized by a high reactivity, which however confers instability in field application.

To overcome this problem we synthesized a series of derivatives with the hydroxyl groups protected to obtain prodrugs of the natural compound.

The seven derivatives obtained were characterized by different protecting groups, ranging from a simple alkyl chain up to aromatic structures increasingly conjugated.

The synthetic procedure had given good results for each derivative except for **ESP7**: in this case it was not possible to protect the hydroxyl group in position 5 on the bicycle chroman-4-one.

Furthermore, **ESP4** and **ESP5** were obtained with low yield (27.70% and 33.60%, respectively).

In terms of antioxidant capacity, Hesperidin was found to have a powerful radical-scavenger ability with broad spectrum of action, it had been shown to be active against three different radicals. All synthetic derivatives showed no antioxidant activity, demonstrating the fact that activity of the natural compound resides in the phenol in position 2 on bycycle croman-4-one, which was protected in all the obtained derivatives.

Regarding antifungal activity, both Hesperidin that its derivatives were not able to inhibit the growth of dermatophytes; in fact the highest inhibition was observed for derivatives **ESP1** and **ESP3** at the concentration of 100 μm/ml on *Epidermophyton floccosum*, with inhibition values equal to 10 and 15%, respectively.

These results allowed us to assume that synthetic derivatives may be pro-drugs of Hesperidin: they improve its applicability with best chemical-physical characteristics and they should not be able to modify its biological properties.

Finally, all the synthetic derivatives were found to be stable in cosmetic formulations compared to the formulation containing the active natural ingredient.

5.3 Salicin and Baicalin derivatives

The synthesis of the derivative of Salicin, **SA1**, was characterized by a lot of positive aspects, such as simplicity of procedure, not higher costs and high yield.

Regarding the antifungal activity, Salicin and its semisynthetic derivative **SA1**, showed low percentages of inhibition although, the derivative **SA1** gave inhibition values of 24.31% and 32.04% at the two assayed concentrations on *Microsporum gypseum*.

Simultaneously to the study of synthetic derivatives of the natural molecule with the aim of improving stability and bioavailability in topical formulations, it was decided to investigate the use of polymers as drugs encapsulation systems. We started to study this new approach with Salicin and Baicailin.

For the inclusion of Salicin, we used three different types of polymethyl metacrilate (**PMMA**) with different particle sizes and absorption capacity: **PMMA-H**, **PMMA-PH**, **PMMA-P**. Each complex was tested and in particular we obtained different results from the Dissolution test data: **Salicin-PMMA-H** complex showed the most rapid release, in fact, after only 5 minutes 100% of Salicin was in solution in the buffer, while **Salicin-PMMA-P** and **Salicin-PMMA-PH** complexes have reached 100%, respectively, after about 10 and 20 minutes.

As regards Baicalin, the synthesis of its derivative (**B1**) gave us the same result in terms of synthetic procedure in comparison with **SA1**. The acetylation reaction has allowed to obtain a stable lipophilic derivative, as demonstrated by *in vitro* analysis. The antioxidant activity

was indeed found to be higher for Baicalin than **B1** that showed a very low activity because of all the phenolic functionality were protected.

None of them can be considered as a potential antifungal molecules but, the semisynthetic derivative **B1** showed higher inhibition values compared to the starting molecule on *Trichophyton violaceum*, *Epidermophyton floccosum* and *Trichophyton tonsurans*.

B1 maintained the pharmacological activity of the starting product and improved the stability of the active ingredient in the formulation.

In this case, only **PMMA-H** (that had greater absorption capacity) was chosen to study the Drug-polymer complex. **Baicalin-PMMA-H** complex was successively analyzed by dissolution test: Baicalin was dissolved in the buffer solution after only 5 minutes.

These preliminary data, although they were not so positive, led us to investigate what was the real problem: the capacity of the polymer to be loaded, the inclusion tecnique, the chemical and physical nature of the active molecule used or if the polymer was not able to contain drugs inside.

5.4 Drug- PMMA-H: inclusion and release

New methods of inclusion in polymethyl methacrylate polymers have been studied. **PMMA-H** was selected because more absorbent than the other polymers (**PMMA-PH** and **PMMA-P**).

Two loading ways were selected: one for hydrophilic compounds (using Ethanol) and another one for lipophilic ones (using an oil phase).

Oils chosen were Vaseline, synthetic oil with a sufficiently high melting point that, once used for the production of the complex, remained as integral part of its, and HMDS, a silicon with thw ability to evaporate at room temperature.

Although the two methods were quite different, and for the chemical-physical properties of **PMMA** was expected to get better results the loading technique that use the oil phase, both of inclusion methods had proved to be optimal with yields never less than 95%.

Different molecules were chosen for this research: Benzydamine Hydrochloride (**BZD**), Glycyrrethinic Acid (**GLA**) and Quercetin (**QE)** for Ethanol's tecnique and Ibuprofen (**IBU**) for oil tecnique. Each of complexes obtained gave very positive yields and percentages of inclusion.

First complexes obtained (**BZD-PMMA-H** and **IBU-PMMA-H** loaded in Vaseline and HMDS) were analyzed by DT to test if actives were in the **PMMA-H** core.

Dissolution curves, both in the case of **BZD-PMMA-H** and **IBU-PMMA-H**, had given good results: the drug was released from the polymer and the dissolution curves of the complexed drugs were lower than the dissolution of free drug in all cases investigated. These results had two positive effects: it was a proof that the drug had been loaded into the polymer matrix and was not only sticked to the polymer surface, in addition, the release from the polymer to the dissolution medium was to be gradual.

These results allowed us to affirm that two very effective and transverse loading methods had been identified; they could be used with the majority of drugs and cosmetic actives because it depends only on their hydro / lipophilicity; the fact that the polymer were able not only to absorb the drug but also to release the drug, allows **PMMA** as possible carrier in formulation.

Topical formulation was our focus, so obtained complexes were placed in different formulations (gels, lipogel, W/O and O/W emulsions) to ensure that, once in the formulation, the release of drugs from the complex was different compared to the release of free drug in the same formulation. For the release assessment, Franz cells had been used and each analysis was been repeated at T0 and after 30, 60 and 90 days.

Release analysis at T0 that gave the best result was **BZD-PMMA-H** in the Gel: although **BZD** was very soluble in the water, it required a lot of time toleave from the polymer core because its release was very low.

Then, T30, T60 and T90 release curves in the presence or in the absence of the polymer were equal to each other.

The situation was different when, **BZD-PMMA-H** complex was put in a cream: the two releases were already the same at T0.

Also in the case of **IBU-PMMA-H** complex in Lipogel, there was not difference in the release between **IBU-PMMA-H** in Vaseline and free **IBU** while, at T0, the curve of **IBU-PMMA-H** (HMDS) was slightly higher. Over time, the three curves were uniform.

W/O and O/W emulsions with **GLA-PMMA-H** showed different behavior: a lower release in W/O formulation containing the complex was foun, compared to the same

formulation with free **GLA**, but it was more evident only at T0. In the O/W formulations the two curves were comparable since the first analysis.

We can therefore say that the most common topical formulations (gel, lipogel and emulsions) were not correct vehicles to obtain the controlled release performance of the polymer. **PMMA-H** could not hold the drug in the formulation, in a receiving medium where it is very soluble. In the presence of an oily phase in the formulation, the ability to retain the drug inside decreased because **PMMA-H** probably prefered to exchange the oil phase present in the formulation with the drug. However, considering that the isolated complex performances were better, future studies could be made on the use of complexes in pressurized systems or patches for topical application.

QE-PMMA-H complex was added to cosmetic formulation W/O and O/W emulsion in order to verify if the polymer was able to protect the active ingredient from degradation. Quercetin was chosen because is a flavonoid with antioxidant activity and had stability problems in the formulation. The ability of **PMMA-H** to protect Quercetin was monitored using an antioxidant test. The stability was evaluated under accelerated aging at 40 °C in 90 days. In both cases (W/O and O/W formulations) the polymer had defended Quercetin only for the 10%, probably this was the percentage of the molecule that remained inside **PMMA-H** after 90 days. In the case of O/W emulsion, **PMMA-H** reduced the rancidity of Quercetin: in the O/W emulsion with free **QE** that turned brown after 90 days, in contrast to the same formulation with the polymer. This result showed that **PMMA-H** was able to improve the stability and the organoleptic properties of an active ingredient in a topical formulation.

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