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Propolis for *Herpes simplex* lesions: Review of the evidence and design of a lipstick for its application

Final degree project

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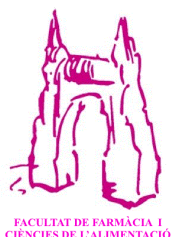
Principal field: Pharmacognosy and phytotherapy

Secondary fields: Pharmaceutical technology and microbiology

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1. Abbreviations

HSV: *Herpes simplex Virus*

UVB: Ultraviolet B Radiation

ACV: Acyclovir

ACF: Antiviral Complex of Flavonoids

INCI: International Nomenclature of Cosmetic Ingredients

PG: Propylene Glycol

q.s.p.: quantity sufficient per

q.s.: quantity sufficient

HPLC: High Performance Liquid Chromatography

GC-MS: Gas Chromatography – Mass Spectrometry

UV-VIS: Ultraviolet – Visible Light

PG: Propolis Group

AG: Acyclovir Group

DER: Drug Extract Ratio

2. Context

On one hand, I always have been seen herpes lesions: at the family, friends, work, at the street... and I'm afraid of number of people that are suffering of it. Sometimes those affections are little, but other times people get covered with a big painful lesion that affects to their mood and social behaviour and lead the person to the desperation and to following non-scientifically verified tips. On the other hand, I heard that propolis could be an effective way to treat herpes and it sound interesting to me.

Personally, I believe that bee products properties are interesting on medicine, but I cannot ignore "anti-solutions" that humans realize in desperation, so my proposal was to verify if propolis could be a remedy for cold-stores or not, and if it was right, try to do something useful with that information.

3. Objectives

- To verify if propolis could be or not effective to treat herpes lesions.
 - To know which propolis compounds are responsible for its effect.
 - To know propolis mechanism of action.
 - To know if there are a group of propolis more effective to treat herpes lesions than others.
- To make a fast superficial investigation at the market searching if there are some propolis lipsticks commercialized.
- To elaborate a formulation proposal of a propolis-based cosmetic.
 - To prepare a propolis extract suitable for its incorporation into the cosmetic proposal.
 - To design a lipstick formulation and optimise it for its organoleptic properties.

4. Abstract

Propolis is a natural substance produced by bees that show equal or more efficacy than acyclovir on herpes lesions. Mechanism of the antiviral action seems to be blocking the virus entrance into the cell. In addition, other actions related to relieving associated symptomatology like pain, tension and burning sensation, are of interest. Some major constituents present in propolis have been identified as active, such as galangin, chrysin, pinocembrin, benzoic acid, caffeic acid and coumaric acid. Nevertheless, the activity of the isolated compounds was ever lower than for the mixture of the propolis constituents. Some of the most used propolis extracts in antiviral activity experimentation with herpes lesions are ethanolic extracts and aqueous extracts. They differ in composition, action time and efficacy, despite in both cases efficacy in front placebo and acyclovir was demonstrated.

On the market, different propolis lip balms could be found, but no propolis lipstick, making it a commercial opportunity. In order to design a propolis lipstick proposal, a propolis extraction with propylene glycol, was prepared and used on the lipstick. Final formula take into account different needs of a person who suffers from cold-sore as a little bit of sun protection, nutriment, hide the lesion and have a good sensation at the organoleptic level.

5. Resum

La pròpolis és una substància natural produïda per les abelles que ha demostrat ser igual o més eficaç que l'aciclovir en les lesions herpètiques. El mecanisme d'acció antiviral sembla ser que és el bloqueig de l'entrada del virus a la cèl·lula. Addicionalment, altres accions relacionades a l'alleujament de la simptomatologia com el dolor, la tensió i cremor, són d'interès. Alguns constituents majoritaris de la pròpolis s'han identificat com a actius, com per exemple la galangina, la crisina, la pinocembrina, l'àcid benzoic, l'àcid cafeic i l'àcid cumàric. Ara bé, l'acció dels components aïllats sempre és menor que la barreja dels constituents de la pròpolis. Alguns dels extractes de pròpolis més utilitzats per a dur a terme l'experimentació de l'activitat antiviral en lesions herpètiques són els extractes etanòlics i els extractes aquosos. Aquests difereixen en composició i temps d'acció i eficàcia, tot i que s'ha demostrat, en ambdós casos, la seva eficàcia davant del placebo i de l'aciclovir.

Al mercat, s'han trobat diversos bàlsams labials amb pròpolis, però no pintallavis amb pròpolis, cosa que fa que aquest pugui ser una oportunitat comercial. Per tal de fer una proposta de pintallavis de pròpolis, s'ha preparat un extracte de pròpolis amb propilenglicol, que s'ha utilitzat com a ingredient del pintallavis. La fórmula final té en compte diferents necessitats de la persona que pateix de panses herpètiques com és una mica de protecció solar, nutrició, amagar la lesió i tenir una bona sensació a nivell organolèptic.

6. Field integration

Pharmacognosy and phytotherapy: Because of propolis have a natural origin, the substance is a study field of pharmacognosy. In that project, different parameters of propolis are explained as composition, viral activity, how to prepare an extract and medicinal doses.

Pharmaceutical technology: Formulation concepts are a base to create a lipstick as well to understand the function of each ingredient.

Microbiology: Know how *Herpes simplex* viruses interact with humans is essential to understand other related information.

7. Introduction

7.1. Herpes simplex virus

The infection with *Herpes simplex virus*, world-renowned as “herpes” and almost exclusive of humans, can be due to *Herpes simplex virus type I (HSV-1)* or *Herpes simplex virus type 2 (HSV-2)*, which share a big number of characteristics. HSV can produce lytic infections on mucus-epithelial cells and latent infections on neurones with no detectable lesion. There are different stimulus able to produce a recurrence of the infection such as stress, fever or sunlight (UVB). If the stimulus are enough, viral replication will turn on at neurons, making the viruses able to move about nerve and finally cause lesions on the same dermatome. In general, recurrent infections are less intense, shorter and more localized than the original ones thanks to immunologic memory (1,2).

An infected patient will be a source of infection during the entire life and secretions (saliva, liquid from vesicles and vaginal secretions) on direct contact is the only way to transmit HSV. The two types of herpes viruses can produce genital and oral lesions although is more common to contract HSV-1 at oral-labial zones and HSV-2 at genitals (figure 1) (1).

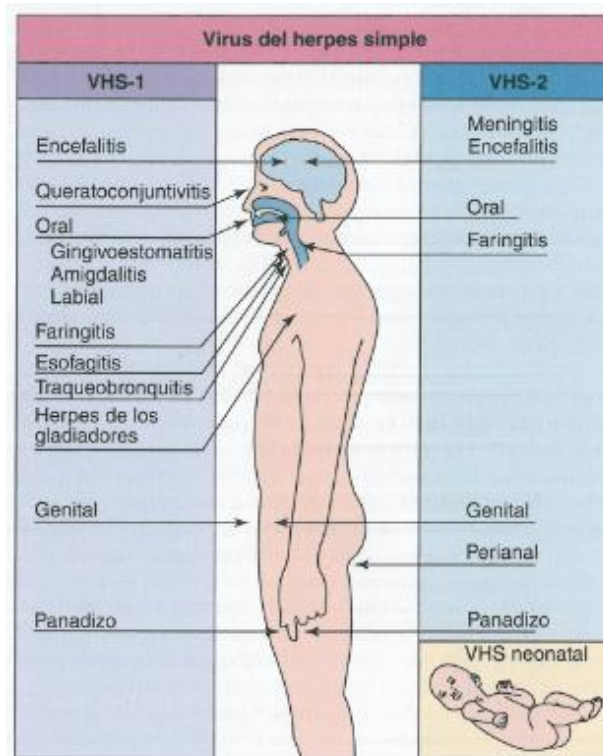


Figure 1

Common infection locations for HSV

From: Microbiología médica (1)

Active herpes infections symptoms are tingling, itch, burning sensation, pain and general symptoms like headache, fever, swollen lymph nodes, flu-like symptoms, etc. Sometimes that symptoms could appear few days before the lesions (2,3). The classical development of a herpes lesion is a grouped or single transparent blister on top of an erythema that progress to a pustular lesion, ulcers, scabs (only on dry surfaces) and finally healing in a gap of 14-28 days (1,3,4).

Some possible complications for HSV are severe diseases such as encephalitis or keratitis that can occur to immunocompromised people, neonatal herpes, higher risk of acquire HIV and/or express a more severe HIV infection (2).

The main treatment for HSV infection is acyclovir (ACV), but also valacyclovir, pencyclovir and famcyclovir are widely used and have a similar mechanism of action. Nowadays it doesn't exist a vaccine to prevent or treat HSV (1,2). ACV on his behalf is a nucleotide analogous that inhibits viral DNA synthesis, and make a selective action on infected cells by being transformed into acyclovir-monophosphate by a viral thymidine kinase (5).

Administration of acyclovir topical cream at a concentration of 50 mg/g is useful to relieve the local symptomatology and reduce the duration of the acute infection (3,5). The adverse reactions are not usual but can occur; some of them are burning or itching after applying the cream and dryness and descale of the lips as the most popular (6). It is surprising that there are no clinically significant problems of resistance between immunocompetent hosts to the current treatments but then, in immunocompromised patients, rates of resistance varies from 4% to 14% (7).

7.2. Propolis

The word "propolis" derives from ancient Greek that means "defence of the hive" making reference to the function of that product on the hive (blocking the cracks, sealing spaces, protect larvae, protect from microorganisms...) (8,9). Propolis use is not recent given that Egyptian, Greek and Roman are some examples of civilizations that used the substance (9).

Raw propolis is composed about of 50% resin of plant origin, 30% of bee wax, 10% aromatic oils, 5% of pollen and 5% of other substances. That mixture result in a lipophilic, hard and brittle material that turns into a very sticky, gummy, pliable and soft material at temperatures from 25°C to 45°C. When reaches temperatures of 60°C-70°C most of the propolis will became liquid. It possess a pleasant smell and a colour that usually ranges from yellow to dark brown (10). Usually, propolis is harvested in autumn by manual extraction and there are two ways for collecting that material:

a) The traditional way consist on scratching the surfaces of the hive when other materials like honey or wax are picked up (figure 2). This is a low productive process and collected propolis must be cleaned because usually includes excrements, dead bees and other contaminants. To clean this propolis it can be water washed and manually cleaned by collecting big elements like dead bees, or also can be cleaned by covering propolis with water and heat at high temperature (more over 90°C) for hours. With this process, melted wax, wood impurities and other materials will float at the surface.

b) The other way to harvest propolis consist in introduce on the top of the hive a “propolis trap” (that is a piece of plastic or net with holes) placed in a way that air, light and non-desirable elements for bees could enter on the hive. With this method, bees tend to place propolis in the propolis trap in order to protect the hive (figure 3). To collect the propolis, the trap must be removed and frizzed for few hours, and right away folded, punched and scraped. Cooling process is necessary to make propolis a hard and brittle material. That system is much more effective and obtained propolis tend to not contain big impurities amounts, so cleaning process is not necessary (11–14).



Figure 2 (on top)

Propolis attached to the outer surfaces of the hive.

From: Bee culture (14)

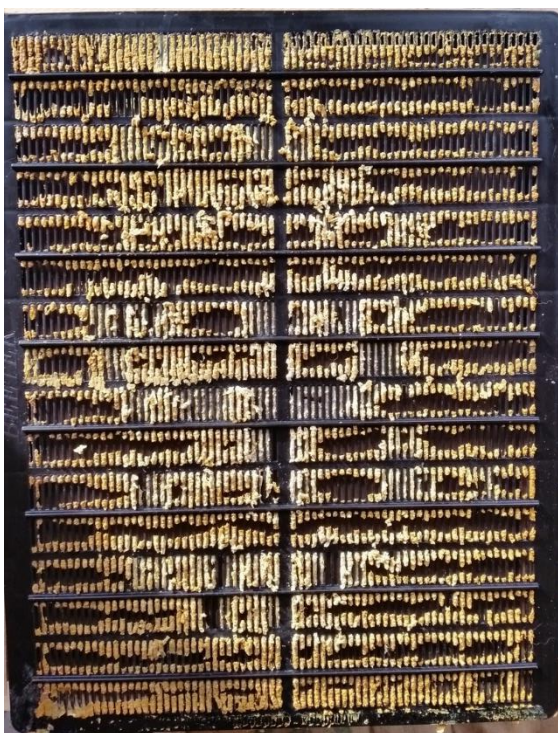


Figure 3

Propolis trapped on a “propolis trap”

From: Business of bees (15)

Chemically, propolis is a not-defined mix of substances; at the moment, more than 300 different components have been identified and classified into more than 180 different groups of chemicals (9). Nevertheless, some compounds are frequently present in the analysed samples of propolis and they are used for its characterization (10). One way to organize the constituents is grouping them on phenols, terpenes, carbohydrates, minerals, vitamins and others (9,16,17):

- **Phenols:** They include flavonoids, phenolic acids, tannins, stilbenes, curcuminoids, and coumarins. These substances are considered responsible for most of propolis properties (anti-oxidant, anti-tumoral and anti-inflammatory, for example). Flavonoids, the most abundant subgroup, include pinocembrin, chrysin, quercetin, and galangin, among others.
- **Terpenes:** There are monoterpenes and sesquiterpenes, such as geraniol, bisabolol, guaiol and farnesol. They provide the characteristic odour to propolis and can be used as markers for quality control. In general, terpenes play a role in antimicrobial activity. Additionally, higher molecular weight terpenes, such as steroids can also be found in propolis.
- **Carbohydrates:** They are mainly monosaccharides (glucose and fructose) and disaccharides (sucrose). Their origin is not yet well known, but is suggested that nectar and honey would be potential sources.
- **Minerals:** Propolis contain useful minerals for humans like magnesium (Mg), calcium (Ca), potassium (K), sodium (Na), zinc (Zn) or iron (Fe), but also can include toxic elements as mercury (Hg) or lead (Pb).
- **Vitamins:** Vitamins E, C, B1, B2 and B6 were found. About vitamin B1 (thiamine) and B2 (riboflavin), they come from pollen. Presence of vitamins has been pointed as relevant in therapeutic properties.
- **Other:** Are fatty acids, aliphatic hydrocarbons, bee enzymes (glucose-6-phosphatase), etc.

The variability of the propolis composition is a difficulty for its standardization; however, different types of propolis have shown similar activities (8).

Propolis extraction could be realized with a wide variability of solvents, among which water, methanol and ethanol are the most popular. The choice of the correct solvent is

relevant because final propolis extract composition will depend of it, as well geographical origin and season of harvesting (8,9). Most of the active ingredients can be extracted with propylene glycol or ethanol; the latter is preferred to obtain low-wax propolis extracts. Water is able to extract few active constituents but extraction capacity can improve by heating (8).

Orally, administrated to mice or humans, propolis have not shown side effects. The safe dose suggested for humans was 1.4 mg/kg/day that means moreover 70 mg/day (50 kg of weight). Topically, few cases were reported of contact dermatitis in beekeepers (8).

Several medicinal properties of propolis have been reported, such anti-bacterial, anti-fungal, anti-protozoal, anti-tumoral, anti-inflammatory, anti-oxidant, hepatoprotective and wound healing activities.

Propolis can act against bacterial infections destroying cell wall and cytoplasm, and stopping protein synthesis. In general, propolis is more effective on gram-positive bacteria than in gram-negative one. The anti-inflammatory activity is due the presence of flavonoids that are able to inhibit leukotriene and prostaglandin production as well to delay some enzyme action like myeloperoxidase activity.

Wound healing capacity is due to immunomodulatory, anti-inflammation, anti-microbial and anti-oxidant activities but also thanks to accelerate cells metabolism, blood circulation and formation of collagen fibres (9).

Nowadays there are two important commercial propolis on market: ethanol Propolis Extract ACF® and Propolis GH 2002 (18,19).

7.3. Lipsticks composition

Lipsticks are anhydrous cosmetics made of a correct mixture of fatty oils, fats, waxes and colouring agents. They are used to give to lips an attractive colour and minimize imperfections (20,21).

To formulate a lipstick, it is fundamental to take into account the possible interactions between ingredients and the consequences of an incorrect proportion of them. The bee wax is one of that fundamental ingredients that could produce some problems: use of beeswax offer plastic properties and improves the compatibility with the most polar used ingredients such castor oil but, in contrast, elevated amounts produce loose of brightness and granulated consistency. A solution for this problem could be combine bee wax with carnauba or candelilla waxes which are able to improve stick shine. Candelilla wax is less brittle than carnauba wax.

Another problematic ingredient is propolis, and it is important to notice that raw propolis is not used on lip balms. Instead of raw propolis, that would probably give a sandy sensation, propolis active constituents could be introduced as a propylene glycol

extract, which is able to keep the activities. To increase formula stability when propylene glycol extract is used, the inclusion of lanoline and high proportions of castor oil might be useful.

Some ingredients are able to perform more than one function at the same time. This is the case of petroleum jelly (paraffinum liquidum) that generate an occlusive stratum and offers shine. The use of petroleum jelly entail risk of a lack of lipstick permanency on lips so adding also squalane, an ingredient able to improve substances penetration on skin, would be a good combination (8,20–24).

A good lipstick should have a great appearance, be innocuous, easy to apply, with a good skin sensation, with a stable colour and able to fix on lips as well as remove of them intentionally (21).

8. Methods

8.1. Literature research

Studies were searched on the databases Scopus, PubMed and Hindawi with the words like “propolis AND herpes”, “propolis AND antiviral”, “propolis AND acyclovir”, “propolis AND properties”. Selected studies were propolis properties reviews and clinical and preclinical studies of herpes treatment with propolis extracts.

8.2. Market research

Market research consists on a key search with the words “propolis lipstick” and “propolis stick” on websites www.amazon.es and www.promofarma.com (25,26). Search also included few results of Google web search engine with the words “propolis lipstick” (27).

Products that differ by the odour or colour, but have the same base formula are considered as one unique result.

On Google web search engine were excluded images or publicity promoted entries.

8.3. Propolis extraction

INCI does not define which solvents are suitable for introducing propolis extracts in a cosmetic formula, even so, it is known that propylene glycol is able to retain the constituents of interest (8,28).

Propylene glycol (PG) is a transparent, colourless, organic and hygroscopic liquid common in cosmetics, drugs (including oral and parental pharmaceutical forms) and

alimentary industry. A topical formulation of 20% or upper concentrations of that substance could produce irritation on humans. Propylene glycol is water miscible but also is able to dissolve essential oils (29).

Solubility of propylene glycol in *Ricinus communis* seed oil was tested in order to verify their compatibility.

The propolis used in the present work was purchased from a local beekeeper that produces his products on Setcases, Catalonia, Spain.

For the preparation of the extract, the patent US4382886 (30) was used as a guide but with some modifications. In any case, an extract with a drug-extract ratio of approx. 1:2 was obtained. In the present work, 9 g of raw propolis chopped into fine splinters were extracted with 36 mL of propylene glycol in a closed amber flask for 10 days, shaking at least 3 times per day and protected from light. After extraction, content was filtered to remove solids. After filtration the extract was evaporated at a temperature of 95 °C (± 15°C) to approximately 18 mL.

8.4. Lipstick design

To realize the formulation a pattern formula was used and tested several times with little modifications in order to transform it into the wanted lipstick. All ingredients used were safe and easy to get. On table 1 two pattern formulas were exposed (24,31).

Pattern formula A		Pattern formula B	
Castor oil	q.s.p. 50 g	Almond oil	q.s.p. 50 g
Pigments	10 g	Shea butter	13 g
Jojoba oil	8 g	Beeswax	10 g
Carnauba wax	5 g	Castor oil	8 g
Beeswax	4 g	Cacao butter	5 g
Squalane	3.5 g	Pigments	1.5 g
Titanium dioxide ^a	2 g	Vitamin E	q.s.
Vitamin E	q.s.		

Table 1

Two pattern formulas (A and B) adjusted a 50g of mass. Common ingredients share same colour. ^{a)} Titanium dioxide is considered also a pigment.

The equipment used in preparation of the formula was: electronic scale with a precision of 0.01 g, hot plate, aluminium lipstick mould, mortar, filters, thermometer, fridge, and glass labware.

Preparation process take into account some critical points (21–23):

1. Waxes need to be melted as first ingredients and before adding oils and soft ingredients (due to the highest melting points). This will avoid excess of temperature on more thermo sensible ingredients.
2. Final mixture could be reheated to mix it properly or to get enough temperature to pour into moulds or cases without solidifying during the process.
3. Vitamin E needs to be added at the end of the process, due to be thermo sensible.
4. Pigments could create lumps so use of mortar or filters may be necessary.
5. Just after lipstick starts solidification process, on the mould, it is recommended to move the lipstick to the fridge and let it chill for some minutes.

Tests of the different formulas were performed using stick lip balm plastic cases until an appropriate solid consistency was reached, allowing the use of the lipstick mould. The used aluminium mould needs to be lubricated, before pouring lipstick mass, with paraffinum liquidum.

During the process, all real weights and incidences would be registered. Organoleptic properties assay consist on visual, touch and olfactory personal sensations.

9. Results

9.1. Pre-clinical evidence

Five studies were selected and compared (19,32–35). All studies show in-vitro results and realized a chemical determination of their extracts (table 2).

All studies have showed similar results (table 3) despite using propolis from different origins and preparing their extracts in diverse ways. Used extracts were aqueous or ethanolic and were compared with acyclovir and heparin as a positive control, and also with other isolated propolis compounds (Benzoic acid, caffeic acid, chlorogenic acid, chrysin, galangin, *p*-coumaric acid, pinocembrin and quercetin).

The isolated substances galangin, chrysin, caffeic acid and chlorogenic acid showed anti-HSV-1 activity but ever minor than propolis extracts, demonstrating synergy between propolis compounds on antiviral activity.

Studies demonstrated that propolis extracts are highly effective to block virus entrance to the cell, showing viral inhibition when applied during absorption phase or when is used as viral pre-treatment (incubate virus with propolis before entering in contact with cells) in front of acyclovir; in opposition, low or null efficacy is observed when propolis is used as a cell-pre-treatment or during replication phase.

Propolis extracts have a time and concentration dependent effect, the ethanol extract is more active but requires more time to realize the same action than aqueous extract (figure 4 and 5).

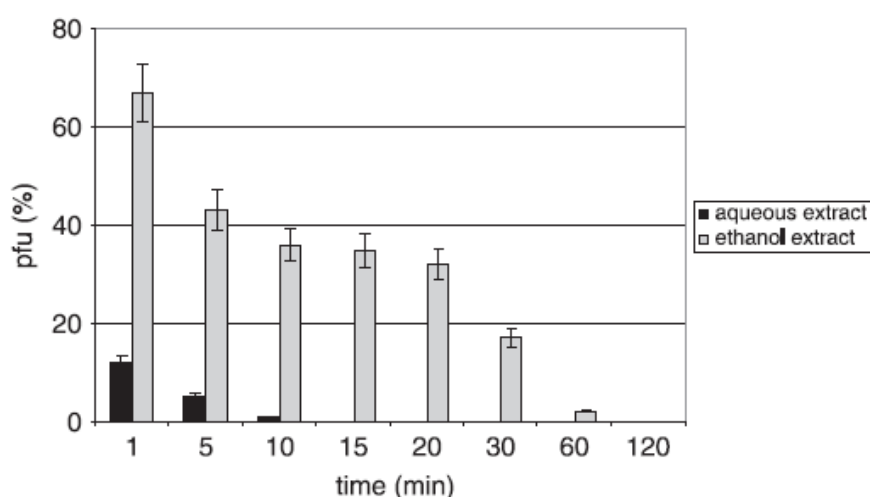


Figure 4

Time dependence of an aqueous and ethanol extract in front of HSV-1

From: Paul Schnitzler et al., 2009 (32)

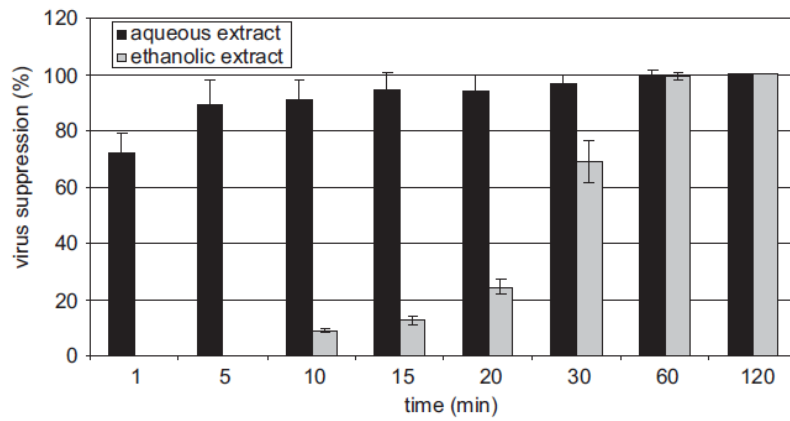


Figure 5

Time dependence of an aqueous and ethanol extract in front of HSV-2

From: Silke Nolkemper et al., 2010 (34)

The ethanol extracts are less effective at cold temperatures (4°C) than at room temperature, and does not show big antiviral activity differences between HSV-1 and HSV-2.

Synergy over replication phase is demonstrated between ethanol extract and acyclovir, being HSV-1 more sensitive.

	Propolis extract	Analytical technique	Results
Paul Schnitzler <i>et al.</i> , 2009 (32) and Silke Nolkemper <i>et al.</i> , 2010 (34)	Aqueous extract of propolis from Moravia (Czech Republic) and ethanol extract of propolis GH 2002	HPLC	In both extracts, main components are polyphenols, flavonoids and phenylcarboxylic acids. Differences between extracts could be found on flavonoids (qualitatively and quantitatively) and in phenylcarboxylic acids (quantitatively). Ethanol extract is rich in flavonoids (chrysin, pinocembrin and galangin). Aqueous extract very low in flavonoids but have higher levels of phenylcarboxylic acids (benzoic acid, cinnamic acid, caffeic acid and <i>p</i> -coumaric acid).
V. Bankova <i>et al.</i> , 2014 (19)	Propolis extract ACF®, batch SW-21	GC-MS	Compounds that showed concentrations of more than 2.00% were benzoic acid, coumaric acid, ferulic acid, caffeic acid (aromatic acids), benzyl <i>p</i> -coumarate (ester), pinocembrin and galangin (flavonoids), pinocembrin chalcone (chalcones) and hexoses.
Ayşe Yildirim <i>et al.</i> , 2016 (33)	Ethanol extract of propolis collected from Hatay region (south of Turkey)	HPLC	The four most abundant compounds (in decreasing order) were galangin, chrysin, caffeic acid phenethyl ester (CAPE) and pinocembrin.
Abbas Hazem <i>et al.</i> , 2017 (35)	Aqueous extract, and ethanol extract using residual propolis fraction of water extraction	UV-VIS spectrophotometry	Spectrophotometric assay of: <ul style="list-style-type: none"> - Total polyphenols expressed as caffeic acid/chlorogenic acid - Flavonoids expressed as rutin/queracetin Content of polyphenols and flavonoids is higher in the ethanol extract than in the aqueous extract.

Table 2

Chemical determination of ethanolic and aqueous extracts used to test antiviral (HSV) activity on pre-clinical studies of table 3.

	Propolis extract	Positive control	Other tested substances	Cells	HSV type	Results
Paul Schnitzler <i>et al.</i> , 2009 (32)	Aqueous extract of propolis from Moravia (Czech Republic) and ethanol extract of propolis GH 2002 Aqueous extract of propolis from Moravia (Czech Republic) and ethanol extract of propolis GH 2002	Heparin-Na and acyclovir	Caffeic acid, <i>p</i> -coumaric acid, benzoic acid, galangin, pinocembrin and chrysin	RC-37 cells	HSV-1	<ul style="list-style-type: none"> - Galangin and chrysin are the most effective substances when applied as virus pre-treatment. - Efficacy of both extracts was superior than isolated compounds in virus pre-treatment with a reduction of 98% virus infectivity due to the synergy of activities. - Propolis extracts do not produce a significant inhibitory effect if are applied as cell pre-treatment or during viral replication. - Both propolis extracts have a concentration and time dependent effect being ethanol extract 10 times more active and 8 times slowly than aqueous extract. - Required times to practically abolish virus infectivity is about 15 minutes for the aqueous extract and 120 minutes for the ethanol extract.
Silke Nolkemper <i>et al.</i> , 2010 (34)		Acyclovir	---		HSV-2	<ul style="list-style-type: none"> - Both extracts nearly abolish virus infectivity after 60 minutes of virus pre-treatment. - Extracts do not show efficacy in cell pre-treatment or in viral replication. - Extracts show a concentration and time-dependant effect.

V. Bankova <i>et al.</i> , 2014 (19)	Propolis extract ACF®, batch SW-21	Acyclovir	---	MDBK cells	HSV-1 and HSV-2	<ul style="list-style-type: none"> - Extract show antiviral activity during adsorption phase and viral pre-treatment. - Virucidal effect is approximately equal for two HSV types. - At 4°C virucidal effect is lower than at room temperature. - Most effective concentrations start at 10 mg/mL.
Ayşe Yildirim <i>et al.</i> , 2016 (33)	Ethanol extract of propolis collected from Hatay region (south of Turkey)	Acyclovir	---	HEp-2 cell line	HSV-1 and HSV-2	<ul style="list-style-type: none"> - Extract is less effective on replication phase than acyclovir in both HSV types. - When extract is applied on replication phase, there is a gap of 24 hours for HSV-1 and 48 hours for HSV-2 before inhibition effect starts. - On replication phase propolis extract and acyclovir showed significant synergy, which was more evident on HSV-1 than on HSV-2.
Abbas Hazem <i>et al.</i> , 2017 (35)	Aqueous extract, and ethanol extract using residual propolis fraction of water extraction	Acyclovir	Quercetin, caffeic acid and chlorogenic acid	HeLa ATCC® CCL-2™, MG63 ATCC® CRL-1427™ cells	HSV-1	<ul style="list-style-type: none"> - Extracts-virus direct contact is equal efficient as acyclovir for alcohol extracts, caffeic acid and chlorogenic acid. - The antiviral effect is weak or non-effective if compounds or extracts were applied as cell pre- or post- infection treatment.

Table 3

Resume of the antiviral effects of propolis extracts or propolis isolated compounds in front HSV in in-vitro experiments.

9.2. Clinical evidence

Three studies were compared, two with labial lesion and one with genital lesion. All of three studies showed similar results and evaluated healing and symptomatology releasing.

9.2.1. A comparative multi-centre study of the efficacy of propolis, acyclovir and placebo in the treatment of genital herpes (HSV) (36)

- **Propolis extract:** 78-85% ACF propolis-ethanol extract.
- **Condition:** Recurrent chronic genital affection caused by HSV-2.
- **Participants:** 46 men and 44 women from 18 to 69 years without comorbidity. Participants do not show a herpes episode, taken medical treatment of antibiotics, corticoids or immunotherapy, were pregnant or in an intended pregnancy programme during the last 30 days.
- **Studied parameters:** Healing velocity and relief of symptomatology.
- **Study duration:** 10 days.
- **Posology:** 4 times per day on infected and surrounding area.
- **Study design:** Randomized, multi-centre and single-blind (doctors). People was divided into 3 groups: tested substance group (3% propolis ointment), positive control group (5% acyclovir ointment) and negative control group (pH-neutral placebo ointment with propolis vehicle). Some participants start observation with vesicular stage and others with ulcerated stage. Controls were done at days 3, 7 and 10 of the experiment.
- **Results:**
 - On 3rd day, more people propolis group had no symptomatology (itching, burning, pain, paraesthesia, etc.) than in acyclovir ($p = 0.069$) and placebo ($p = 0.042$). On 7th and 10th day no participant reported symptoms.
 - At the end of the treatment, more patients were healed in propolis group than in acyclovir and placebo group ($p = 0.0015$). Details are shown on figure 6.

Stage at control		Initial stage					
		vesicular			ulcerated		
		propolis	acyclovir	placebo	propolis	acyclovir	placebo
Initial stage	n	12	15	20	18	15	10
day 3	vesicles	5	9	9	0	0	2
	ulceration	7	5	11	3	8	8
	crusts	0	1	0	15	7	0
	healed	0	0	0	0	0	0
day 7	ulceration	7	11	15	0	2	2
	crusts	5	4	5	8	9	5
	healed	0	0	0	10	4	3
day 10	ulceration	0	1	5	0	0	1
	crusts	6	12	10	0	3	2
	healed	6	2	5	18	12	7

Figure 6

Healing stage in each study group at days 3, 7 and 10 of the experiment.

From: N. Vynogard et al., 2000 (36)

9.2.2. Comparative study with a lip balm containing 0.5% propolis special extract GH 2002 versus 5% acyclovir cream in patients with herpes labialis in the papular/erythematous stage: A single-blind, randomized, two-arm study (18)

- **Propolis extract:** Propolis extract GH 2002 (2:1 ethanol extract). 78-85% ACF propolis-ethanol extract.
- **Condition:** Herpetical affection on lips (no recognition test was realized to claim HSV type 1).
- **Participants:** 375 participants of both genders from 18 to 70 years on erythematous or papular stage herpes lesion and who suffered at least 4 previous episodes. Were excluded patients with hypersensitivity to some component used in formulas, patients with concomitant viral infections, patients who suffer from immunodeficiency, and patients with a severe herpes labialis which require systemic treatment.
- **Studied parameters:** Healing velocity, relief of symptomatology and global treatment assessment.
- **Study duration:** 10 days.

- **Posology:** 5 times per day (every 3-5 hours) on entire lips.
- **Study design:** Randomized, multi-centre and single-blind (doctors). People was divided into 2 groups: tested substance group (0.5% propolis extract lip balm) and positive control group (5% acyclovir ointment). Controls were done at days 0, 2, 3, 4 and 5. Additional controls were realized for non-healed patients on days 8 (\pm 1 day) and 10.
- **Results:**
 - Less study participants developed vesicles or erosions ($p < 0.0001$ in both cases) if were treated with propolis (figure 7 for more information).
 - Propolis are able to complete encrustation or epithelisation with a 0.5 days of difference ($p < 0.0001$).
 - On propolis group relief of symptomatology (pain, itching, swelling, tension and burning) is significant ($p < 0.001$).
 - Propolis treatment receive a very good impression of efficacy ($p < 0.0001$).
 - The advantages of propolis over acyclovir were especially detected within first 3-5 days after the beginning of treatment.

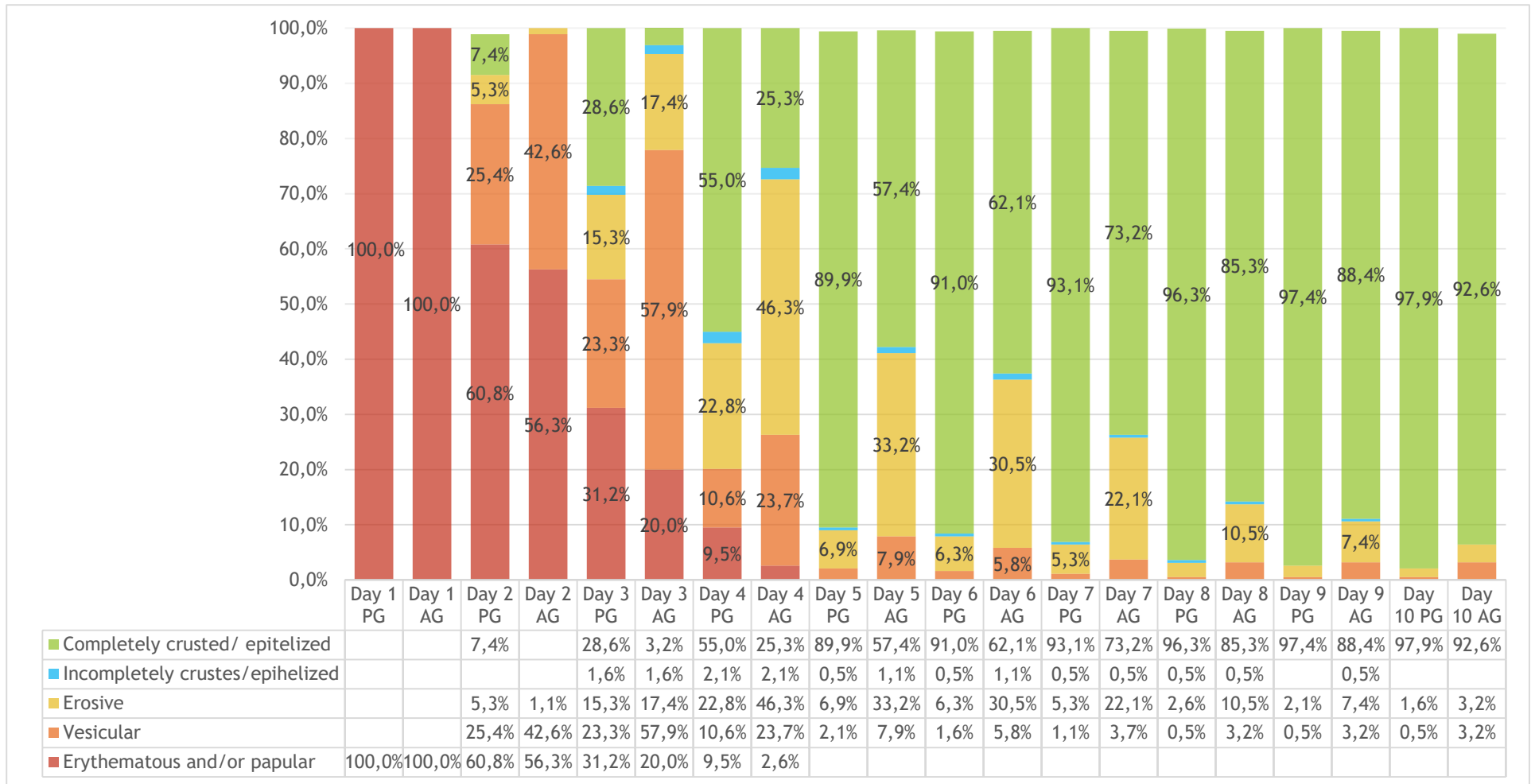


Figure 7

Healing stage of the propolis group (PG) and of the acyclovir group (AG)

From: MD. Petr Arenberger et al., 2018 (18) with adjustments

9.2.3. Lip creams with propolis special extract GH 2002 0.5% versus acyclovir 5.0% for herpes labialis (vesicular stage) (37)

- **Propolis extract:** Propolis extract GH 2002 (2:1 ethanol extract).
- **Condition:** Herpetical affection on lips (no recognition test was realized to claim HSV type 1).
- **Participants:** 397 participants of both genders from 18 to 80 years on vesicular stage herpes lesion and who suffered at least 4 previous episodes. Were excluded patients with hypersensitivity to some component used in formulas, patients with concomitant viral infections, patients who suffer from immunodeficiency, and patients with a severe herpes labialis which require systemic treatment.
- **Studied parameters:** Healing velocity, relief of symptomatology and global treatment assessment.
- **Study duration:** 10 days.
- **Posology:** 5 times per day (every 3-5 hours) on entire lips.
- **Study design:** Randomized, multi-centre and double-blind. People was divided into 2 groups: tested substance group (0.5% propolis extract lip cream) and positive control group (5% acyclovir cream). Controls were done at days 2, 3 4 and 5. Additional controls were realized for non-healed patients on days 7 (± 1 day), 9 and 10.
- **Results:**
 - Propolis treatment demonstrates to be faster healing the lesions than acyclovir treatment ($p < 0.0001$).
 - Pain reduction with propolis treatment was specially stand out between days 0-2 ($p < 0.01$)
 - Symptoms like itching, burning, tension and swelling were noticeably reduced with propolis treatment from the 2nd to the 5th days. But more evident on the 2nd day ($p < 0.00001$).
 - Propolis treatment was considered more efficient than acyclovir treatment ($p < 0.00001$)

9.3. Market products

Market research results are reflected on table 4. One Google result was eliminated to be already present on Promofarma results.

	Keyword	Results	Colour	Available brand
Amazon	propolis lipstick	1	Yes	No
	propolis lip balm	10	No	Yes
Promofarma	propolis lipstick	0		
	propolis lip balm	1	No	Yes
Google	propolis lipstick	2	Yes	No
		5	No	Yes

Table 4

Market research results. Available brand refers if some referenced producer could be identified on Google search engine (finding references of same or other propolis related products).

9.4. Propolis extract

Pre-filtered propolis extract was a dense, dark and opaque mass difficult to filter (filtration takes more than 8 hours). The filtered extract could be defined as a transparent intense amber colour and fluid liquid with no special odour.

For the evaporation of moreover half of filtered extract, two hours were required; major part of the process was realized under magnetic agitation and dissolvent lose was controlled by weight. When evaporation process was finished some propylene glycol needs to be added to the mixture to adjust drug extract ratio to 1:2 (grams of drug for total extract millilitres).

The 1:2 extract was fluent as the original extract, but colour turned from light to dark amber colour.

9.5. Lipstick formulation process

The two pattern formulas (table 1) were tested to decide which of them is more adequate to use as a pattern formula. Pattern formula A was realized on first place using a mortar to improve pigment dispersion and neither the method (more than 10% losses and no appreciable improvements) nor formula were considered adequate.

Pattern formula B was selected to perform the experimentation (test number 1). Several modifications were introduced in 4 additional tests, giving a total of 5 tests. Formula and modifications in each test are shown in table 5 and explained below.

Test 1: (pattern formula): Consistency was good and release an oily layer easily which is good for hydration but could be easily be disgusting, pigment concentration was very low and although obtaining a coloured lipstick it is not able to release a covering layer. Brightness is null and some fine cracks appeared on the bar.

Test 2: Consisted in testing the introduction of propylene glycol (propolis extract solvent) and make other modifications to the formula to prevent compound separation and to readjust consistency. Results were a good incorporation of the propylene glycol and a big improvement on tactile sensation (more creamy). Consistency needs better adjust (it was too tender: between an ointment and a lipstick) and cracks turned more evident and deep. Mate appearance and coloration still needs to solve.

Test 3: Several modifications were done in order to solvent the problems observed after test 2. The amounts of some fats were decreased, a part of beeswax was changed by candelilla wax and pigment proportion were triplicated. On test 3 gross filtration was introduced when oily mixture was introduced into waxy mixture to eliminate big agglomerations of pigment. Corrections were effective but pigment, glow and hardness could still grow and needing of a perfume was considerate.

Test 4: Petroleum jelly, squalane, rose hip oil and perfume were incorporated. Increment of red pigments made lipstick able to covering properly the skin. Test 4 was poured on the moulds.

Test 5: (final formula): The only difference with test 4 was propolis (propylene glycol was replaced by propolis extract). No differences were appreciated between test 4 and test

5. Some fine imperfections were still be observed on the surface of the lipstick but are not appreciable by touch. On figure 8, test 5 could be observed.



Figure 8

Test number 5 (propolis lipstick)

Test number	1	2	3	4	5
Almond oil	q.s.p. 50	q.s.p. 50	q.s.p. 50	q.s.p. 50	q.s.p. 50
Castor oil	8	10	11	11	11
Rosehip oil				1	1
Shea butter	13	9	8	7	7
Cacao butter	5	5	6	6	6
Beeswax	10	11	6	6	6
Candelilla wax			5	6	6
Petroleum jelly				1	1
Lanoline		1.5	1.5	1.5	1.5
Squalane				0.5	0.5
Propylene glycol		3	3	3	
Propolis extract					3
Vitamin E	q.s.	q.s.	q.s.	q.s.	q.s.
Red pigments	1	1	3	4	4
Titanium dioxide	0.5	0.5	0.5	0.5	0.5
Perfume				q.s.	q.s.

	Ingredient increases
	Ingredient decreases
	New ingredient

Table 5

Summary of each test composition and ingredient relation with precedent test proportion.

10. Discussion

10.1. Effectiveness of propolis extracts in front herpetic lesions

Studies about antiviral effects of propolis in front HSV were recent, being the oldest analysed study from 2000 (36) and the newest from 2018 (18,37). Although not finding big discrepancies between the studies (18,19,32–37), is necessary continue with antiviral activity experimentation and propolis active constituents identification due to lack of information in different aspects, such as the possible synergy with acyclovir in vivo, the way of preparation of a most effective propolis extract and the mechanism of action of the extract.

The facts that ethanol extracts contains more and higher levels of flavonoids than aqueous extracts, and that they are also more active is probably related, but is not clear why aqueous extracts have a faster action, considering that phenylcarboxylic acids amounts are higher in aqueous extracts (32,34).

Some substances like galangin, chrysin, caffeic acid, and chlorogenic acid, naturally present in propolis, demonstrated antiviral activity on two separated studies (32,35), but apart from realize a minor effect when tested individually than reference propolis extracts, there are discrepancies on caffeic acid antiviral activity, the only constituent tested by the two studies. Anyway, all discrepancies between all the analysed studies (18,19,32–37) are focused on the same one, which has a chemical determination of both by total polyphenols or total flavonoids amounts. That article (35), point out propolis extracts as equal effective treatment than acyclovir for HSV, as opposed to all others articles that admit propolis antiviral activity better than acyclovir in at least one phase of viral replication.

Ayse Yildirim *et al.*, 2016 (33) showed that propolis was less effective than acyclovir on replication phase; that is not a contradiction because is supported also by other articles (19,32,34,35) and is known that acyclovir acts precisely on replication phase (5).

The only three clinical studies found (18,36,37) although a gap of time of 18 years and being focused on different body areas (genital and oral), have a very similar results among them and point out propolis as a faster way than acyclovir to heal herpes lesions. Comparison of the three studies is limited, due to different studied parameters and participants inclusion criteria: N. Vynogard *et al.*, 2000 (36) checked HSV type on the participants but included less patients, do not make an exclusion criteria according to the number of herpes previous episodes and neither evaluated symptomatology relief or global treatment assessment.

Relief of symptomatology, produced by propolis treatment, (18,36,37) is according to medicinal properties on review studies (9) and probably is one of the factors that make participants think that propolis treatment is more effective than acyclovir treatment.

As in the case of acyclovir, better results with the propolis treatment would be obtained if is applied on the earlier affection stages (18,36,37).

10.2. Market situation

It's important to notice that coloured propolis formulation were found only in the webpages Amazon and Aliexpress, were description errors are common.

In front of non-coloured propolis formulation, coloured products are scant and producers cannot be identified clearly. It generates mistrust on this products because it is not possible to access basic information such as ingredients or verify if lipsticks really include propolis.

All that information makes notice that propolis lipsticks are not common or, more probably, inexistent in Spanish market, making a propolis lipstick an interesting market opportunity.

10.3. Evaluation of formula design

Formula design included formulation and extraction process. About that last, will be interesting use powdered raw propolis (propolis needs to be frozen to be pulverized) (38) which could offer the possibility of realize directly a 1:2 extraction. To use raw propolis would be necessary to know correct granulometry to avoid filter obstruction.

Formulation result was focused on organoleptic properties and still need to know stability (temperature and time resistance, possible compound separation, oxidation, odour changes, etc.) as well to realize antiviral activity tests.

Propolis extract proportion on lipstick is about 3%, according to N. Vynogard *et al.*, 2000 (36) article, but probably propolis proportion could decrease keeping it's activity due to MD. Petr Arenberger *et al.*, 2018 (18) and Jagienka Jautová *et al.*, 2018 (37) used 0.5% propolis extract proportion and also were the only two studies that tested propolis in vivo on the lip area. Selected concentration was 3% to claim enough propolis and also due to the difficulty of formulate with the propolis extract; it means that little imperfections noticed on surface probably would be avoided or minimized reducing propolis extract proportion. Another option to reduce imperfections will be use a more concentrated extract, with the risk of having an important change on rheological

properties of it, or continue adjusting ingredients proportion, a process that probably will require several attempts and time.

Pigment agglomeration is an important point to treat (also obtaining a homogeneous layer when lipstick is applied). According to Wilkinson *et al.*, 1990 (21) is necessary to work pigments with some type of mill (ex. ball mill); on little scale, manual mortar will be correct but produces big loses of material and use of propylene glycol seems to improve a lot pigment dispersion. In that case, filtration process do not produces big loses and is much more effective preventing lumps on the final product; anyway, is important to continue searching how to minimize material loses in that conditions.

11. Conclusions

- Propolis is equal or more effective than acyclovir to treat herpes lesions.
- Efficacy of propolis is not only due to his antiviral properties but also his capacity to relief symptomatology.
- Propolis antiviral mechanism of action against HSV is not well known but presents efficacy on adsorption phase and in virus direct contact.
- Propolis constituents work in synergy. Galangin, chrysin, pinocembrin, benzoic acid, caffeic acid and coumaric acid are considered the most relevant.
- Different propolis types do not show big differences on HSV antiviral activity. It's more relevant how propolis is picked up and how extract is prepared.
- Propolis extract is suitable in cosmetics although INCI do not specifies allowed dissolvents and neither is necessary to declare it.
- No propolis lipstick is commercialized in Spain. Some propolis lip balms could be found but they do not claim any antiviral activity nor propolis extract proportion.
- A lipstick formula containing 3% of a propolis propylene glycol extract (DER 1:2) has been designed and prepared. It shows good organoleptic properties.

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