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Vývoj a fyzikální stabilita polotuhého přípravku s rostlinným extraktem

Developement and physical stability of semisolid fomulation with plant extract

Jitka Malečková

Vedoucí diplomové práce: Doc. RNDr. Pavel Doležal, CSc.
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Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

Jitka Malečková

CONTENTS

SOUHRN

Práce se zabývá vývojem prakticky použitelného přípravku typu topického gelu s využitím rostlinného extraktu obsahujícího flavonoidy a polyfenoly. Hlavními cílem bylo vytvořit fyzikálně a funkčně stálý externě aplikovatelný polotuhý přípravek. Funkční stabilita rostlinného extraktu byla hodnocena před a po provedení stabilitních zkoušek, a to metodou stanovení volného radikálu 1,1-diphenyl-2-pikrylhydrazyl (DPPH•).

Teoretická část diplomové práce stručně shrnuje základy o polotuhých přípravcích, se zaměřením na gely, především na gely karbomerů. Je zmíněno i využití topicky aplikovatelných polotuhých forem s antioxidačními vlastnostmi. Zahrnuty jsou také metody a principy vyhodnocení fyzikální stability pomocí texturní analýzy, střídáním teplot, centrifugace, změny barvy, vibračního testu, fotostabilitních testů a testů funkční stability (DPPH•) přípravku.

Experimentální čast popisuje metodiku určení obsahu fenolů a dílčí kroky vývoje konečného přípravku, jakými byly hodnocení vlivu oxidu titaničitého a množství karbomeru na texturní vlastnosti a pH přípravku. Hodnocení fyzikálních stability přípravků bylo prováděno vizuálně, pouze texturní analýza přístrojově. Funkční stabilita byla hodnocena pomocí metodologie DPPH•.

Výsledky testů dokazují vysokou antioxidační aktivitu extraktu z C. sativa, a tudíž hlavní předpoklad pro inkorporaci do topických přípravků. Finální přípravek, který byl podroben jak stabilitním tak funkčním zkouškám, se až na ukazatele fotostability ukázal být stálý.

ABSTRACT

An investigation presented was concern in development of a practically usable topical gel having incorporated the plant extracts containing flavonoids and polyphenols to stabilize the formulation. The functional stability of this plant extract before and after stability tests were evaluated using the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH•) assays.

A theoretical part of the thesis briefly summarizes basics about semisolid formulations focused in gels, namely carbomers, and the use of the topical formulation possesing antioxidant activity.

An evaluation of the physical stability by texture analysis, temperature cycling test, centrifugation, change in colour, vibration and photostability test as well as functional stability (DPPH•) is also included in this part.

An experimental part describes the techniques of determination of the total amount of phenolic compounds, preparation of the final formulation and evaluation of the influence of titanium dioxide and concentration of carbomer on texture properties and pH. Excepting texture analysis, determination of the physical stability was mostly performed with a visual evaluation, functional stability was determined with DPPH methodology.

The results show high antioxidant activity of the extract and ability to use it for topical formulations. The final formulation which has been submitted to physical and functional testing was shown to be stable in all test except the photostability parameter.

1 PREFACE

This thesis was worked out on the Division of Pharmaceutical Technology of Pharmaceutical Faculty, University of Porto and was linked to the research which is in progress at this Department.

The development of the final formulation was the main and longer–term part of this research.

Lyophilisation of the extract and determination of total phenolics were done by I. F. Almeida and were performed in the Faculty of Engineering, University of Porto.

Photostability testing was performed in Bial, pharmaceutical company in Portugal, also by I. F. Almeida.

2 INTRODUCTION

2.1 Topical dosage forms

For skin care and the topical treatment of dermatological diseases, a wide choice of vehicles ranging from solid to semisolids and liquid preparations is available to clinicians, patients and consumers [1].

Semisolid preparations include therapeutic ointments, creams, pastes, gels and other forms with similar consistency intended for application on the skin. Semisolids may serve as vehicles for topically applied drugs, as emollients or as a protective or occlusive dressings on the skin [2].

According to the European pharmacopeia (5^{th} Ed) several categories of topical semi-solid preparation may be distinguished.

Ointments are semisolid preparations that are intended to be applied externally to the skin or mucous membranes. They consist of single-phase basis in which solids or liquids may be dispersed. Ointments are divided in Hydrophobic (lipophilic) ointments, Water-Emulsifying Ointments and Hydrophilic Ointments. **Hydrophobic Ointments** can absorb only a small amount of water. Typical bases used for their formulation are hard, liquid and light liquid paraffins, vegetable oils, animal fats, synthetic glycerides, waxes and liquid polyalkylsiloxanes. **Water-Emulsifying Ointments** can absorb larger amounts of water and thereby produce water-in-oil emulsions depending on the nature of the emulsifiers: water-in-oil emulsifying agents such as wool alcohols, sorbitan esters, monoglycerides and fatty alcohols, or oil-in-water emulsifying agents such as sulphated fatty alcohols, polysorbates, macrogol cetostearyl ether or esters of fatty acide with macrogols maybe used for this purpose. Their bases are those of the hydrophobic ointments. **Hydrophilic Ointments** are preparations having bases that are miscible with water. The bases usually consist of mixtures of liquid and solid macrogols (polyethylene glycols). They may contain appropriate amounts of water.

Creams are multiphase preparations consisting of a lipophilic phase and an aqueous phase. **Hydrophobic Creams** have as the continuous phase the lipophilic phase. They contain water-in-oil emulsifying agents such as wool fat, sorbitan esters and monoglycerides**. Hydrophilic Creams** have as the continuous phase the aqueous phase. They contain oil-in-water emulsifying agents such as sodium or triethanolamin soaps, sulphated fatty alcohols and polysorbates, combined, if necessary, with water-in-oil emulsifying agents.

Pastes are semisolid preparations containing large proportions of solids finely dispersed in the bases.

2.2 Gels

Within the major groups of semisolid preparations, the use of gels has expanded, both in cosmetics and pharmaceuticals [1]. Gels are excellent drug delivery system for various routes of administration and are compatible with many different drug substances [2].

According to the United States Pharmacopeia (USP28/NF23) gels are semisolid systems of either suspensions made up of small inorganic particles or large organic molecules interpenetrated by a liquid. Where the gel mass consists of a network of small discrete particles, the gel is classified as a two-phase system (eg. Aluminum Hydroxide Gel). In a two-phase system if the particle size of the dispersed phase is relatively large, the gel mass is sometimes referred to as magma (eg. Bentonite Magma). Both gels and magmas may be thixotropic, forming semisolids on standing and becoming liquid on agitation [3].

Single–phase gels consist of organic macromolecules distributed uniformly throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid. Single-phase may be made from synthetic macromolecules (eg. carbomer) or from natural gums (eg. Tragacanth) [3].

Gel systems can be also divided into Hydrophobic Gels and Hydrophilic Gels. **Hydrophobic gels** (oleogels) are preparations whose bases usually consist of liquid paraffin with polyethylene or fatty oils gelled with colloidal silica or aluminium or zinc soaps. **Hydrophilic gels** (hydrogels) are preparations whose bases usually consist of water; glycerol or propyleneglycol gelled with suitable gelling agents such as tragacanth, starch, cellulose derivates, carboxyvinyl polymers and magnesium-aluminium silicates

Gels are semirigid systems in which the movement of the dispersing medium is restricted by an interlacing three-dimensional network of particles or solvated macromolecules of the dispersed phase. A high degree of physical or chemical crosslinking can be involved. The increased viscosity caused by the interlacing and consequential internal friction is responsible for the semisolid state. A gel can consist of twisted, matted strands often wound together by stronger types of van der Waals forces to form crystalline and amorphous regions throughout the system [2].

The physical and chemical properties of the gel are affected by the order of addition of reactants, pH, temperature, concentration of reactants used and conditions of aging of the gel [3].

Out of the various semisolids dosage forms, the gels are becoming more popular due to ease of application and better percutaneous absorption than other semisolid preparations. Gels can resist the physiological stress caused by skin flexion, blinking and mucocilliary movement, adopting the shape of the applied area, and controlling drug release. Effectiveness of topical application mainly depends upon its rate, and extent of drug release from the base [1]. For development of our formulation we chose as a gelling agent cabomer (Carbopol® 940).

2.2.1 Carbomers

Carbomers have been used mainly in liquid or semi-solid pharmaceutical formulations, such as gels, suspensions and emulsions, as a thickening and viscosity agent, in order to modify the flow characteristics. Recently, they have been used for their mucoadhesive properties and a relevant amount of work has been done on the bioadhesive potential of carbomer polymers [4].

Carbomers are synthetic high - molecular - weight polymers of acrylic acid (**Figure 1**) that are crosslinked with either allyl sucrose or allyl ethers of pentaerytritol (**Figure 2**). They contain between 56% and 68% of carboxylic acid (-COOH) groups calculated on the dry basis.

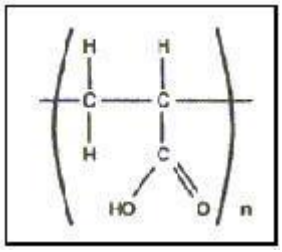


Figure 1: General structure of carbomer polymers.

Carbomer gels are usually prepared by dispersing the polymer in water, followed by neutralization with a base. The repulsion of adjacent ionized carboxylic acid moieties is responsible for the gel formation (**Figure 2**). Gelation can also be achieved in hydrophilic solvents, primarily due to hydrogen bonding between the carboxyl groups of the polymer and the hydroxyl groups of the solvent [5].

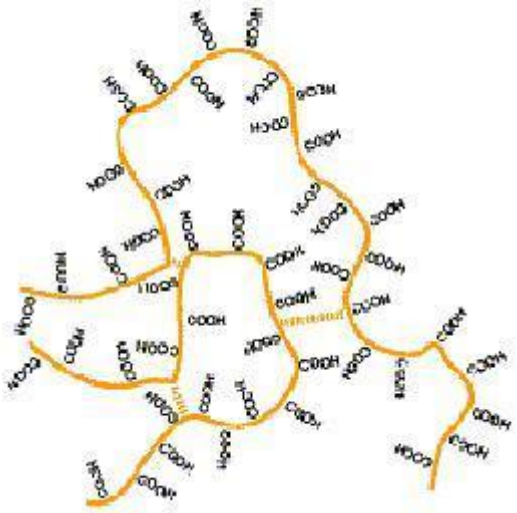


Figure 2: Schematic drawing of a molecular segment of a cross-linked polyacrylic acid polymer

Carbomer polymers have a potential wide range of applicability in the pharmaceutical and dermocosmetic fields. Some advantages of using aqueous carbomer gels are: [6]

- high viscosity at low concentration
- wide viscosity interval and characteristic flow behaviour
- compatibility with many active ingredients
- bioadhesive properties
- good thermal stability
- excellent organoleptic characteristics
- good patient acceptance

The physical properties of the carbomer gels, the time they remain on the application area, and the drug release rates are extremely sensitive to the rheological behaviour of the topical formulations. Therefore, exhaustive characterization of the flow behaviour of these systems as a function of neutralization and polymer concentration is essential to evaluate the ability of

carbomer polymers to jellify for a range of pH values and their potential uses as dermatological bases[7].

2.3 Topical application of plant extract with antioxidant activity

The use of plant extracts in cosmetic formulations is increasing. A cosmetic formulation, may include actives of strictly natural origin, designed to protect the skin against exogenous or endogenous harmful agents, as well as to balance again the dermal homeostasis lipids altered by dermatosis and ageing [9].

The protective effects of plant products are due to the presence of several components. Some are enzymes and proteins and others are low molecular weight compounds such as vitamins, carotenoids, flavonoids, anthocyanins and other phenolic compounds [8]. Most plant extracts that scavenge free radicals contain phenolic derivates. The number and location of the phenolic hydroxyl group on these chemicals are important factors determining the level of scavenging activity [9].

Antioxidants from natural sources may provide new possibilities for the treatment and prevention of oxidative stress-mediated diseases, such as the ones developed in skin. Therefore, in recent years, a lot of researches have been performed, aiming at establish and characterize natural antioxidants to be applied topically [10].

The primary environmental factor that causes human skin aging is UV irradiation from the sun. Changes due to aging in the skin, in which degenerative changes exceed regenerative changes, are characterized by thinning and wrinkling of the epidermis. It is caused by changes in the underlying dermis by loosing of collagen and elastin fibres, with lessened support of epidermal layers, and lessened circulatory perfusion [9].

Topical natural antioxidants are a useful strategy for the prevention of photoaging and oxidative stress mediated skin diseases. In view of this underlying principle, the screening of natural plant extracts with scavenging

activity for pro-oxidant reactive species is a primary requirement for the development of new topical antioxidant formulations.

2.4 Castanea sativa (Fagaceae)

Chestnut (Castanea sativa, Fagaceae) is a species of chestnut native to southeastern Europe and Asia Minor. Chestnut leaves are used in traditional medicine in the treatment of numerous diseases such as bronchitis and cough [11]. Scavenging activity against $O_2^{\cdot -}$ and $HO^{\cdot}$ was previously reported for Castanea sativa leaves and was ascribed to the presence of polyphenols [12].

Figure 3: Castanea sativa – Chestnut fruit, flower and leaves

The development of a topical semisolid formulation including the C. sativa leaf extract was the aim of this work.

2.5 Evaluation of the stability of semisolid formulation with antioxidant activity

Throughout the life cycle products may be exposed to a number of different conditions during their storage, transport, retail and finally use by the consumer.

A stability program should reflect the most likely conditions that will be experienced and attempt to replicate them. Possible areas to be considered may be temperature, light, or physical effects [15].

Besides presenting suitable physical stability, it is important that the actives incorporated in the formulations retain their activity.

2.5.1 Evaluation of physical stability

The industrial protocols (International Conference on Harmonization-ICH) foresee the storage of samples at different temperatures (room temperature and a higher temperature). Temperature has an important effect on viscosity. The temperature dependence of liquids viscosity has been correlated with intermolecular bonding similarities. Under accelerated stability conditions, some systems can undergo phase transitions, and consequently there is a lack of correlation between the behaviour at room temperature and at high temperature. These accelerated tests are of limited use and the formulator faces a challenge for stability prediction [16].

Cycling tests

Tests under conditions which are periodically changed, by imposing changing stresses on a pack, can reveal inadequacies more quickly than can storage at a constant temperature[16]. As products can be expected to encounter

temperature and pressure extremes during transport and storage, stability testing at these extremes should be considered, for example: Low-temperature testing and High-temperature testing[15].

Mechanical tests (Vibration test)

Vibration tests on a suitable vibrator for a period of some hours should be carried out in appropriate instances[16]. Mechanical shock testing is often conducted in order to determine whether shipping movements may damage the product and/or its packaging.

Centrifugation

Centrifugation has wide application in pharmaceutical laboratories for predicting stability of semisolids. The force of gravity acts on the sample making the particles move within it. The centrifuging test produces stress in the sample, simulating an increase in the force of gravity and increasing the mobility of the particles thus anticipating possible instabilities. These changes may appear in the form of precipitation, separation of phases, caking or coalescence among others. The sample is centrifuged at a standardized temperature, time and speed. Afterwards, the sample is visually evaluated [26].

Photostability

Products whose packaging may allow the contains to be exposed to light should undergo light stability testing [15]. The effects of exposure to light are difficult to accelerate in the laboratory. The source of illumination should ideally have the same spectral distribution as daylight. Most artificial light sources do not, although xenon discharge lamps do [16].

Evaluation of the textural properties

In stability tests, the samples are periodically checked for changes in important features. In this work the textural properties were evaluated. Texture can be regarded as a manifestation of the rheological properties of a product. It is an important attribute that affects processing and handling, shelf-life and consumer acceptance of products. Formulations which have been designed for topical application must exhibit acceptable mechanical characteristics e.g. ease of application and low firmness. Textural analysis is widely used for the mechanical characterization of food products and it has also been used in the pharmaceutical and cosmetic areas [17, 18].

2.5.2 Evaluation of functional stability

DPPH (α,α-diphenyl-β-picrylhydrazil) has widely been used as a substrate to evaluate the antioxidative activity of various samples [19]. DPPH is characterised as a stable free radical by virtue of delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centred at about 520 nm. When the solution of DPPH is mixed with a substance that can donate a hydrogen atom, then gives rise to the reduced form with loss of this violet colour.

Primary reaction is:

Z· + AH = ZH + A·

Z· is representing DPPH radical. **AH** is donor molecule**. ZH** reduced form and **A·** free radical.

This latter radical **A·** will then undergo further reactions which control the overall stochiometry, that is, the number of molecules of DPPH reduced (decolorised) by one molecule of reductant. For interpretation of the results from DPPH method the parameter IC 50 is used. This is defined as the concentration of substrate that causes 50% loss of the DPPH activity (colour) [26].

This methodology has been used to evaluate the functional stability of antioxidants either alone or incorporated in dosage forms [10, 20, 21]. It is noteworthy that this method measures the antioxidant activity of the whole extract taking therefore into account possible synergisms. Furthermore, there is no need for markers which could be troublesome when dealing with complex matrixes like plant extracts.

3 EXPERIMENTAL PART

3.1 Materials and instruments

Carbopol® 940 (Lote KC 626 D4, Shutz, Lisboa, Portugal), Glycerol 85% (Lote 49783, Fluka, Italy), Liquid Paraffin Ph. Eur. (Lote 711126), Titanium dioxide (Lote A3404), Triethanolamin pure 99% (Lote 96873124UO), Methylparaben (all supplied by Jose M. VAZ Pereira, Lisboa, Portugal), pH 7.01 buffer solution ±0.01, pH 4.01 buffer solution ±0.01(HI 7004 Hanna Instrument, Germany), DPPH (Sigma, St. Louis MO, USA, lot 013K1351), Ethanol 96% (Aga, Prior Velho, Portugal). Deonized water was used for the preparation of the gel samples.

Rotavapor RE 111 (Büchi, Switzerland)

UV-VIS Spectrophotometer, V 530 (Jasco, Japan)

pH-meter 691 (Metrohm, Switzerland)

Overhead stirrer RZR 2041 (Heidolph, Germany)

Microplate reader ELX 808 IU (BIO-TEK)

Texturometer TAXT2i (Stable Micro Systems, United Kingdom)

Shaking device, WNB 45 (Memmert, Germany)

SUNTEST XLS + (Atlas, USA)

Minolta Chromameter CR 400 (Konica-Minolta, Japan)

3.1.1 Description

Carbopol® 940 – It is a synthetic high-molecular weight polymer of acrylic acid that is crosslinked with either allyl sucrose or allyl ethers of pentathritol.

Glycerine ($C_3H_8O_3$) - It is used in a wide variety of pharmaceutical formulations including oral, otic, ophthalmic, topical and parenteral because of emollient, plasticizer properties.

Liquid paraffin – Heavy mineral oil is primarily used as an excipient in topical pharmaceutical formulations. It is a mixture of refined liquid saturated aliphatic and cyclic hydrocarbons obtained from petroleum.

Titanium dioxide (TiO_2) – In pharmaceuticals formulations it is used as a white pigment. TiO_2 It is also used in dermatological preparations and cosmetics as sunscreen.

Triethanolamin (2.2´.2´´- nitrilotriethanol) – It is widely used as an alkalizing agent in pharmaceutical formulations.

Methylparaben (methyl-4-hydroxybenzoate) – It is used as an antimicrobial preservative in cosmetic, food products and pharmaceutical formulations.

C. sativa leaves - The leaves were collected in Mirandela, Portugal and dried at room temperature in the dark for three weeks. Voucher specimens were preserved in the laboratory of the Pharmaceutical Technology Department, Faculty of Pharmacy, University of Porto, for further reference.

3.2 Methods

3.2.1 Preparation of the extract

The dried leaves (40 g) were extracted five times with 1000 mL ethanol:water (7:3) solution (15 min., 40ºC, magnetic stirring 2 rpm) and filtered with a glass filter funnel (5–15 µm porosity). The extract was evaporated at 40ºC by Rotavapor 111 under reduced pressure followed by lyophylisation to obtain dry extract.

3.2.2 Characterisation of C. sativa extract

Determination of total phenolics

The amount of total phenolics in the extract was determined using the Folin Ciocalteu colorimetric method, according to a described procedure [19]. Briefly, 1 mL of Folin Ciocalteu reagent was added to 300 μL of extract dissolved in ethanol:water (7:3) solution, followed by the addition of 5 mL of 20% (w/v) sodium carbonate solution. The mixture was made up to 10 mL with water, submitted to thorough shaking and the absorbance was read after 20 min, at 735 nm. The contents are expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract. The measurements were performed in triplicate.

DPPH scavenging assay

The scavenging ability of the extract towards the stable free radical 1, 1-diphenyl-2-picrylhydrazil (DPPH) was performed as a general measure for its radical scavenging activity. DPPH is reduced to the corresponding hydrazine with DPPH signal intensity being inversely related to antioxidant concentration and to the reaction time. Thus, the extract scavenging activity was measured by monitoring its reduction reflected in the decrease in absorbance at 515 nm. according to a described procedure [22] with modifications. Reaction mixtures contained DPPH dissolved in ethanol:water (7:3) solution (190 μM) and the extract at different concentrations dissolved in ethanol:water (7:3) solution in a final volume of 200 μL. After 20 min, the absorbance was measured at 515 nm in a microplate reader. The effects are expressed as the percentual inhibition of the DPPH reduction.

Ascorbic acid was used as positive control. Each study corresponded to three experiments performed in duplicate.

3.2.3 Preparation of the semisolid formulations

Formulations with titanium dioxide

Formulations were prepared in overhead stirrer. A specific amount of carbomer was slowly added to hydroglycerine solution with stirring at 500 rpm for 10 min. Formulation was kept overnight in oven at 20 degrees. After 24 hours the hydrogel was homogenized with stirring at 500 rpm for 5 min. Mixture of titanium dioxide and liquid paraffin was slowly added to carbomer hydrogel with stirring at rpm 700 for 20 minuts. Three formulations were prepared with different concentrations of titanium dioxide (**Table 1**).

3.2.4 Characterisation of the formulation

pH measurement

pH meter was calibrated with buffers pH 4 (pH 4.01 buffer solution ±0.01 pH) and pH 7 (HI 7004 pH 7.01 buffer solution ±0.01). Before performing the measurements, gel formulations were diluted with neutral water which was prepared by adding 0.1M NaOH and 0.1M HCl solutions into deionized water to obtain pH 7.

1g of the formulations was diluted in plastic tubes with 3 mL of neutral water. All pH measurements were preformed using a pH / mV meter with glass electrode.

Textural analysis

The textural analysis was performed in the compression mode in the texturometer by carriyng out a spreadability test (T= 20° °C) using a 45º cone probe. Once the test is started the upper cone (male cone) lowers towards the female cone which holds the sample. After penetrating in the sample it keeps moving until it is at the distance of 2 mm from the surface of the holder. So the probe has moved the distance of 23 mm from its start point.

This test measures the spreadability parameter which occurs in the later part of the test while the product is being squeezed out from between the male and

female cone. During penetration the force value increases until the point of maximum penetration depth. This force value can be taken as the Firmness at that point. From the graphic force versus distance the following parameters were calculated: Maximum Force, Positive area, Minimum Force and Negative area. All measurements were in every case performed in triplicate.

The sample holders for the test were filled and then the surface levelled.

Several formulations were tested to determine the influence of titanium dioxide, the influence of pH and the influence of the concentration of carbomer on the textural properties.

Table 1: Composition of the formulations with titanium dioxide

	Formulation		
Substance	**A**	**B**	**C**
Carbomer	2.00	2.00	2.00
Glycerine 85%	26.98	26.98	26.98
Liquid paraffin	12.93	12.93	12.93
Titanium dioxide	**1.00**	**0.50**	**0.00**
Water	57.09	57.59	58.09

Influence of titanium dioxide concentration on texture properties and pH

Titanium dioxide was used for improving the properties of the gel such as stickiness and colour. For studing the influence of titanium dioxide three basic gel formulations were used which are described in **Table 1**.

Influence of pH on texture properties

Carbomers disperse in water to form acidic colloidal dispersions of low viscosity that, when neutralised produce highly viscous gels. Triethanolamin was used as an alkalyzing agent. The influence of pH on the textural properties of the formulations obtained with different concentration of triethanolamin in combination with different concentrations of carbomer (**Table 3**) was studied, in order to obtain suitable viscosity of the formulation. The results were evaluated as described in 3.2.4 by texture analysis.

To optimize the pH of the formulation from pH 3 to obtain pH around 5 triethanolamin 99% was used. Different concentrations of triethanolamin were used to obtain the most suitable value of the pH (**Table 2**).

The triethanolamin was added into gel formulation in the final step of its preparation. It was added diluted with 10 mL of deionized water and manual mixing. These formulations were tested for texture properties and pH (**Table 10**).

Table 2: Composition of the formulations with triethanolamin and carbomer 2%

	Formulation			
Substance	**D**	**E**	**F**	**G**
Carbomer	**2.00**	**2.00**	**2.00**	**2.00**
Glycerin 85%	26.98	26.98	26.98	26.98
Liquid parafin	12.93	12.93	12.93	12.93
Titanium dioxide	0.50	0.50	0.50	0.50
Triethanolamin	**0.75**	**0.50**	**0.35**	**0.00**

Water	56.84	57.09	57.24	57.59

Influence of Carbomer concentration on textural properties

For this study several formulations were prepared (**Table 2**), to determine influence of different concentrations of carbomer on the textural properties. Concentration 2.0%, 1.0% and 0.5% were used. The results were analysed with texture meter and the pH values of formulations were measured as well.

Six different gels (**Table 3**) were prepared and their pH and texture properties were compared (**Table 5**).

Table 3: Composition of the formulation with titanium dioxide, triethanolamin and carbomer concentration 0.5% and 1.0%

	Formulation					
Substance	**H**	**I**	**J**	**K**	**L**	**M**
Carbomer	**1.00**	**1.00**	**0.50**	**0.50**	**0.50**	**1.00**
Glycerin 85%	26.98	26.98	26.98	26.98	26.98	26.98
Liquid paraffin	12.93	12.93	12.93	12.93	12.93	12.93
Titanium dioxide	0.5	0.5	0.5	0.5	0.5	0.5
Triethanolamine	**0.32**	**0.28**	**0.125**	**0.10**	**0.07**	**0.26**
Water	58.59	58.62	58.965	58.99	59.02	58.33

3.3 Evaluation of the physical and functional stability of the final formulation

Three lots of formulation (**Table 4**) were prepared as follows: Methylparaben was dissolved in water and glycerol mixture by magnetic stirring for 30 minutes with aid of heat (40 ◌C) and allowed to cool. The extract was into the cold

mixture added. A specific amount of carbomer was slowly added in this solution with stirring at 500 rpm for 10 min by overhead stirrer. Formulation was kept overnight in oven 20 ◦C. After 24 hours the hydrogel was homogenizated with stirring at 500 rpm for 5 min. Mixture of titanium dioxide and paraffin liquid was added to carbomer hydrogel with stirring at 700 rpm for 20 min. In the end, triethanolamine was added as solution with 10g of deonized water and mixed manually.

Table 4: Composition of the final formulation

Substance	**Amount (g)**	**Function**
Carbomer	1.50	Gelling agent
Glycerin 85% (w/w)	40.47	Hydrophilic emollient and humectant
Liquid parrafin	19.40	Lipophilic emollient
Titanium dioxide	0.75	Colouring agent
Triethanolamine	0.39	Alkalizing agent
Castanea sativa extract	0.75	Active (antioxidant)
Methylparaben	0.225	Preservative
Water	86.52	Liquid phase

3.4 Physical stability

Three different lots were prepared for testing physical and functional stability.

Temperature cycling

The physical stability was evaluated by submitting the formulations to storage at 4 – 40 ºC changing of temperature per 24 hour. All of the samples were protected from the light with aluminium foil. Samples were collected after 7 days for evaluation of textural properties, pH measurement, antioxidant activity and changes in organoleptic properties such as a colour and odour.

Centrifugation

5 g of each formulation were transferred into plastic tubes and submitted to centrifugation at 3000 rpm for 30 min.

Afterwards, samples were evaluated in terms of phase separation.

Vibration test

5 g of each formulation were weighted into plastic tubes. The tubes were placed in a water bath equipped with a shaking. Conditions for the test were 90 strokes/minutes, temperature 40ºC, duration 24 hours. Afterwards, colour and odour were evaluated and the occurrence of phase separation was checked for.

Evaluation of photostability

2 g of each formulation were spread on the bottom of Petri plates with spatula. Samples of each formulation and one dark control of each formulation (covered with aluminium foil) were tested. Samples were irradiated in a SUNTEST XLS for 22 hours at an irradiance level of 250 W/m^2. This device consists in an

accelerated weathering device that replicates exposures to terrestrial sunlight. Light emitted by a xenon arc lamp source (2200 watt) that passes through certain optical filters closely matches the spectrum of sunlight at the Earth's surface. Samples were exposed side by side with a solution of 2 % quinine hydrochloride solution (chemical actinometer), in accordance to ICH guidelines.

The following parameters were evaluated after performing the above mentioned tests:

pH values

All pH measurements were performed in triplicate as described in 4.2.4. In following tests were compared changes of the pH.

DPPH scavenging activity

The antioxidant activity of these formulatios was evaluated by DPPH method.

In plastic tubes 500 mg of formulation were weight and 10 ml of purified water were added. The samples were mixed thoroughly and sonicated for 3 minutes. The samples were centrifugated at 5000 rpm for 30 minutes and the assay was performed with the supernatant to avoid interference of titanium dioxide. Ethanol 96% was used as a solvent.

Colour

The colour was evaluated with the tristimulus Minolta Chromameter CR 400. With the use of this instrument the sample surface is illuminated by a pulsed xenon arc lamp. The light reflected perpendicular to the surface is collected for a tristimulus colour analysis at 450 nm 560 nm and 600 nm using the L*a*b* colour system as determined by the CIE. In particular, the L*a*b* system is very comprehensible.

The L* parameter expressing colour brightness (varying between a value of 100 for a white surface and 0 for a black surface).

The a* parameter represents changes along a red/green axis with changes from +60 for a red surface to -60 for a green surface.

The b* parameter changes from +60 for a yellow surface to - 60 for a blue surface. Measurements were performed in triplicate on each sample.

Textural properties

The evaluation of the textural properties was performed as mentioned previously in 3.2.4.

4 RESULTS AND DISCUSSION

4.1 Characterisation of the extract

The content of total phenolic substances for Castanea sativa was **252.4 ± 8.1** mg of gallic acid equivalents (GAE) per gram of lyophilized extract. The extract presented a strong activity against DPPH. Value IC 50 was found to be 14.79 ± 0.54 µg/ml.

Ascorbic acid presented an IC 50 of **4.93 ± 0.36** µg/ml.

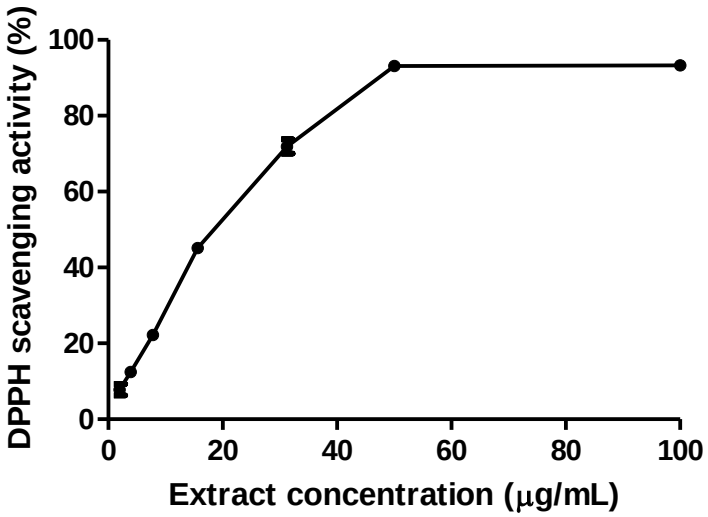


Figure 4: DPPH scavenging activity of the C. sativa leaf extract. The results are expressed as mean ± SEM of three measurements.

The reaction of both vitamin C (as a positive control) and flavonoids with DPPH· is very fast. The high antioxidant activity found for this extract may be due to its high polyphenol and flavonoid contents. In this work, a ethanol:water (7:3) extract from Castanea sativa leaves was used.

Previous work studied an antioxidant activity of the extract from chestnut flower, leaf, skins and fruit. The antioxidant activity was evaluated through several biochemical Assays including DPPH scavenging activity. Among all of the extracts analysed, a significant content of total phenolics (>100 mg/g

of extract, this is more than 10%, for each chestnut compound) and good radical-scavenging activity were found for all extracts, except for fruit. Polyphenols and flavonoids were found in all the samples and in the following order: outer skins - inner skins - flowers - leaves - fruit. It became clear that chestnut leaves, skins and flower present the highest antioxidant activity [27].

Another previous studies demonstrated that this extract presented high effectiveness against reactive oxygen species (ROS) namely superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) as well as on reactive nitrogen species (RNS) namely nitric oxide ($^{\cdot}NO$) and peroxynitrite (ONOO) [28].

A growing body of evidence suggests that skin damage induced by UV irradiation involves the generation of ROS and RNS (Reactive oxygen and nitrogen species) with the consequent oxidative and nitrosative stress, resulting in structural and functional modifications in the cutaneous tissue [13, 14]. Scavenging activity against multiple ROS and RNS seems therefore to be crucial for high effectiveness in the prevention of photo-induced oxidative stress in the skin. Due to the high antioxidant activity of the extract, it is foreseeable that it could be incorporated in topical formulations aiming at protect skin against damage caused by reactive oxygen species.

4.2 Developement of the semisolid formulation

Titanium dioxide was included in the formulation in order to reduce tackiness as well as improve the colour of formulation. It was observed that titanium dioxide concentration did not influence the textural properties of the semisolid formulations (**Table 5**).

Figure 5 represents the maximum force values obtained for formulations with 1.0 and 0.5% of titanium dioxide in comparison with the same

formulation without titanium dioxide. We can clearly see that titanium dioxide did not have any apparent influence on the parameter maximum force. Similar results were found for the other textural parameters.

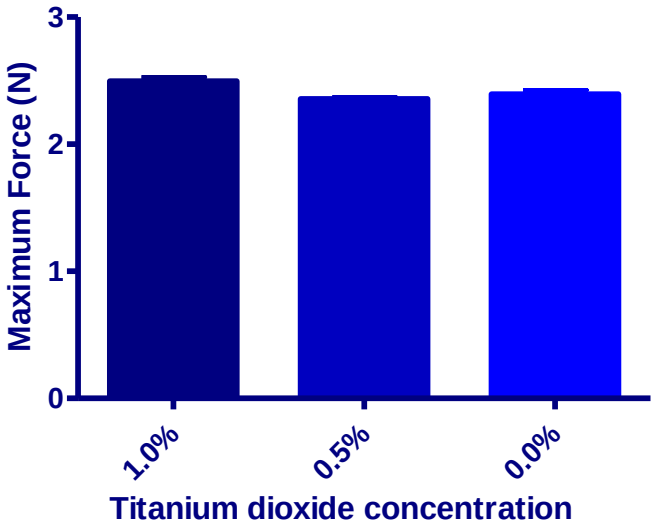


Figure 5: Influence of TiO_2 concentration on the Maximum Force of semisolid formulations (textural analysis was performed at 20ºC).

Table 5: Mechanical properties of formulations obtained with different concentrations of TiO_2 determined by texture profile analysis. Results are expressed as means ± SD (standard deviation).

Formulation	Maximum force (N)	Minimum force (N)	Positive area (N.m)	Negative area (N.m)
A (1.0%)	2.50±0.032	-1.74±0.02	5.91±0.06	-5.14±0.02
B (0.5%)	2.36±0.02	-1.56±0.02	5.66±0.07	-4.90±0.03
C (0.0%)	2.39±0.03	-1.58±0.04	5.76±0.10	-4.91±0.09

Results given in **Table 6** show that different amounts of titanium dioxide did not present any relevant changes to formulations pH**.** As can be see, the formulations pH is acidic which was expected because carbomers form acidic colloidal dispersions (**Table 1**).

The formulations added with carbomer was adjusted to pH around 5 (close to skin pH) by adding triethanolamine as an alkalizing agent. Several formulations were prepared with different amounts of triethanolamine and their textural properties were compared. These studies were performed with the formulation which contained 2% of carbomer. The results of pH are presented in **Table 6**.

Table 6: pH of formulation of 2% carbomer with different amount of triethanolamine

Formulation	D	E	F	G
pH	5.15	4.60	4.45	3.11
Triethanolamine(%)	0.75	0.50	0.35	0.00

Influence of pH on the textural properties of the formulations with 2% Carbomer:

As we can see in **Figure 6**, pH had a great influence on the textural properties. From pH 3.11 (non-neutralized) to pH 4.45 a 4 fold increase was observed for maximum force as well as for the other textural parameters (**Table 7**), which can be attributed to the partial ionization of the carboxylic acid moieties.

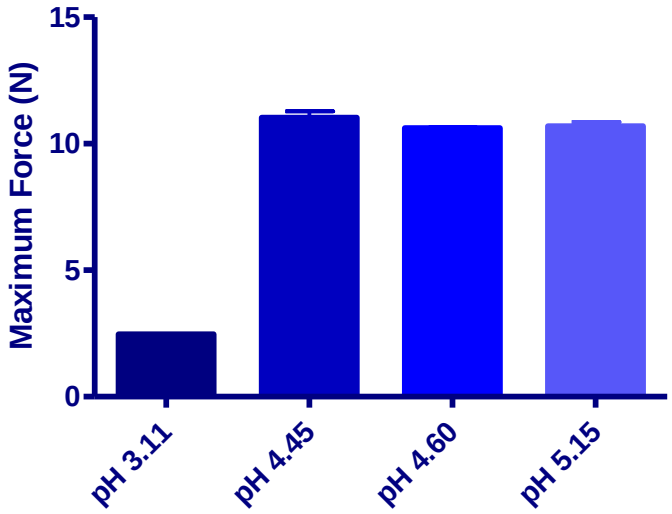


Figure 6: Influence of pH on textural properties of formulations with 2 % of barbomer (T = 20 ºC .

Further increases practically did not produce changes, although we are looking to very small incbeases.

This in accordance with previous work that found that the rheological behaviour of water/g,ycerine/propyleneglycol/Carbopol® 980 eels did not change appreciably in the pH range 5.0-8.0 [7]. On another study, an initial pronounced increase in the consistency index of Carbopol Ultrez 10 hydroalcoholic gels (pH 4-5) was followed for a reduced increment for pH values between 5.5 and 7 I7, 23],

Table '2 Taxtural parameters (mean1SD) of thd formulation with 2% carbomer at diffebent pHq,

Formulation	pH	Maxilum force (N)	Minamum force (N)	@osipive area (N.mm)	Negative area (N.ml)
G	3.11	2.$71 0.01 -1.691 0.00 -1.691 0.00	5&81± 0.05		
@	4,41	11.031 0.24	-6.431 0.15	25.331 .61	-22*72± 1.51
E	4.60	10.421 0.02	-7.07± 0.02	25.02¡0$20	-21.42±-1.13
D	5.15	00.701 0,16	-6.9910.06	2$&841 0.4 -21,88¡-1.13 -21,88¡-1.13	

The aie of this experiment was to develop a fmrmulation with gnmd spreaDing propertiec, appropriate cGnsistencq and pH compatible with application on the skin, The firmnesc ob the fo2mulatiofs with a pH close to skin pH (**Table 8**) was too high which compromised their s`readability. For this reAcon, forlulations with loweb concan4rations of barbomer were prepared.

Table 8: Textural parameters (-e`l 1 SD) of the fmbmulatiols with different carbgmer concentratiofs

r $ Mapiaum force (N) Minimum force (N) Pmsitive area		
force (N)	Minimum force (N)	Pmsitive area (N.m
	10.7010.!6	%6*9910.06 24.(4 0*4 1 -5&41±0.03 1(.981 16.6110.19 **B** 5.22 3.7410.08 24.(4 0*40
.35°0.12 -3.7410.08 10,9810.#0 -10&8710.36		
12 -3.7410.08 10,9810.#0 -10&8710.36	-3.7410.08	10,9810.#0 -10&8710 -10&8710.36

Figure 7 shows t`e iaximqm fmrce of three d-rmulatimnc with diffarent concentratignr nf capbomer (2,0%, 1.0$, .5$) ajd samilap pHs. Adl the textupal paralede`s ifcreasdd with ihcreasing polymer concentbatioj,

EMBED Prism5.Dbcuient 0836

Figure 7: Tex4ural parameter3 maximum force (mean ± SD) of the formulations vith diffebend carbomer conceftradion.

Thiq as indicatafe /f an increace hn the sdrength gf the gel. Dhe fireness of the formudations with 1&0% and 0.5% of carbomer wac considered apprkpraate and the ctudy coltinued by avaluatine the infludnce of pH on theib texttpal prmperties.

According tm this resulds sevaral formulationa of 1. ! `nd 0.$% carboler sith difderent `mounts of triethanolamine were prepabed (see **Ta`la #**) ald **Table 9** presents their pH v`lees.

Table 9: pH of formulat`ons ppepared wath diffebelt amoqft of carbomer and triet`anelamin&

Formuhation

	H	**I**	**J**	**K**	**L**	**L**
pH	**5.11**	**%,08**	**5.22**	**4,88**	**4.61 4 .95** **4.95**	
Tbiedhajmlaeine(g)	0.32	0.08 0 .105 0.105	0.1	0.07		
Barboeer (%)		1.00	1&00	0.50	0.50 0 .%0 0.%0	1.00

The formulatiol **D** (1&0 %(gas qelected because the forlulation wIth 0&5% of carbmmer preqentEd phasd separation w`en subje#ted tO centrifugatiOn (3000 rpm 30 mil.).

4.3 Evaluatigb of `hysical and functional stabilitx

Aycline tamperature tesd Th0ee lods gf the optiiized formulatigl sebe subjected tk cyclIng tesd, where temperature changdd from 4ºC t/ 40²C eve29 24 hours durin' 7 days.

Th0ee lods gf the optiiized formulatigl sebe subjected tk cyclIng tesd, where temperature changdd from 4ºC t/ 40²C eve29 24 hours durin' 7 days.

No changes were observed in dhd colour and odour of the three lcts when compared to a fobmula4ign kept at 208C.

pH values

As ge caf see in the table 10 the pH remaaned unchabged after the cycling test.

Table 102 The pH of the fmrmulationc bedmre and after cycling test. The resuhts are presefted as means ± SD.

	Lot 1	**Lot 2**	**Lot 3**
pH before cycling test	4.7310.05	4.75±0.03	$.7610.03
pH after cycling test	4.74±0. 5	4.7410.03	4.74±0.04

DPPH sc!venging activity

From the **Figure 7**, which showed concentration-dependent activity, obtained for lot 1 after and before cycling test, it was possible to calculate the concentration that show, 50% of antioxidant activity (IC_{50}) by linear regression. Similar results were found for lot 1 and 2. The IC_{50} values found for each lot in the antioxidant assays are shown in **Table 11.**

Table 11: DPPH scavenging activity

	Lot 1 **IC 50** (Mean ± SEM)	**Lot 2** **Ic 50** (Mean ±SEM)	**Lot 3** **Ic 50** (Mean ±SEM)
Before cycling test	17.03 ±1.33	17.72±1.06	17.94±1.11
After cycling test	17.01 ±0.75	17.48±0.93	18.58±0.63

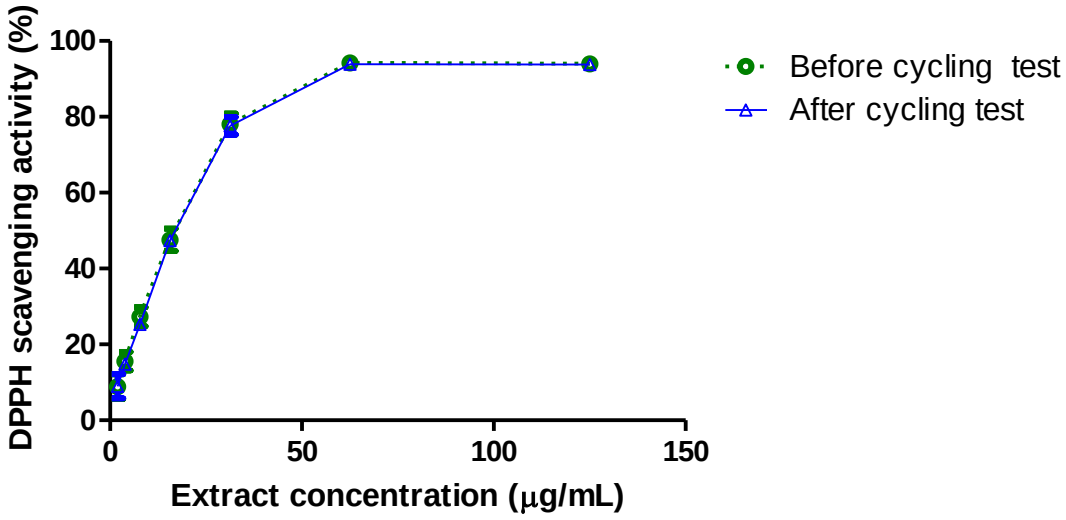


Figure 8: Comparison of DPPH scavenging activity of lot 1 before and after cycling test. Results are expressed as mean ± SEM.

As presented in **Table 11** C_{50} values obtained after cycling test proves the functional stability of the formulation.

The DPPH assay, originally developed by Blois is widely used for the measurement of free radical scavenging capacity in phytotechnology, food technology, and pharmacological toxicology. DPPH is a free radical that

easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It can accommodate a large number of samples within a short period, and is sensitive enough to detect low concentrations of the active principles [21].

The extract formulations tested in the present study for their H-donor ability, measured by the stable free radical DPPH·assay, showed high antioxidant activity.

Texture analysis

After cycling test, no modifications were found for the antioxidant activity what accounts to the functional stability of the studied formulation.

Likewise, the textural properties of the formulation were not modified after being subjected to the cycling test (**Table 12**, **Figure 9**).

For this formulation, the parameters Maximum, Minimum force and Positive and negative area did not change during the 7 day temperature cycling test.

Figure 9 clearly shows that the cycling test did not produce any changes in the parameter maximum force. Similar results were obtained for the other textural parameters (**Table 12**).

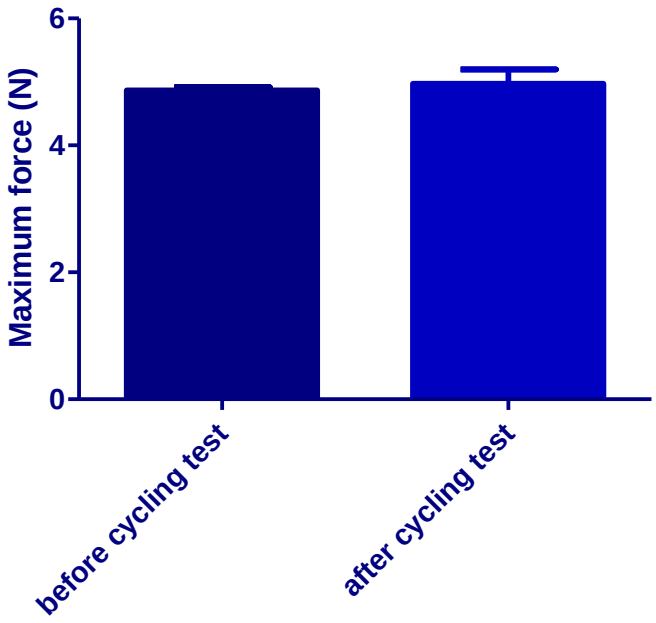


Figure 9: Comparison of maximum force of lot 1 in texture properties before and after cycling test. Results are expressed as means ± SD.

Table 12: Texture analysis of the Lots 1, 2, 3 measured before and after cycling test (Lots 1', 2', 3'). Results Maximum force, Minimum force, Positive and Negative area are expressed as means ± SD.

Formulation	Maximum force (N)	Minimum force (N)	Positive area (N.m)	Negative area (N.m)
Lot 1	4,87±0,05	-3,18±0,05	11,08±0,09	-9,47±0,17
Lot 2	4,78±0,07	-3,02±0,13	11,07±0,14	-9,33±0,20
Lot 3	5,26±0,08	-3,37±0,10	12,14±0,20	-10,39±0,25
Lot 1'	4,97±0,22	-3,21±0,18	11,23±0,30	-9,63±0,38
Lot 2'	4,82±0,14	-3,06±0,08	10,98±0,35	-9,32±0,54
Lot 3'	5,15±0,09	-3,26±0,05	11,74±0,08	-10,12±0,24

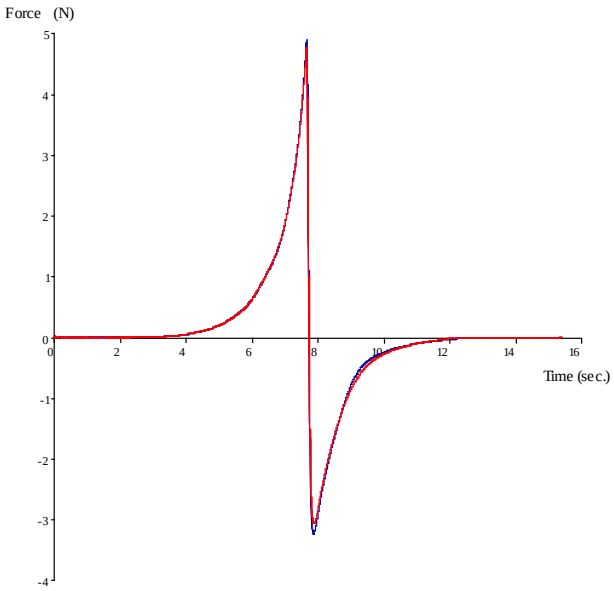


Figure 10: Texturogram

The rheological study allowed to obtain a correct picture of the physical properties and the structural stability of semisolid systems [21]. This study permits to evaluate the textural properties of the different formulations in order to obtain information about physical gels structure and to predict samples behaviour under the physiological conditions as, for example, the application of a stress during sample administration In

fact, examples of textural properties are the ease of sample removal from the container or its spreadability [29].

Centrifugation

In the centrifugation study formulations were stable. We didn´t observe any sign of phase separation.

Figure 11: A test tube with the content of formulation **M** after centrifugation

Vibration test

Vibration test also showed good results. In this case we were looking for modifications especially in organoleptic characteristic such as colour and odour. None presented any modification. Phase separation was not observed as well as changes in colour and odour.

Photostability test:

The formulations found to be unstable to light exposure, in terms of functional stability. DPPH scavenging activity decreased, as IC 50 doubled after the irradiation period (**Table 13).**

Table 13: IC50 of lot 1, 2, 3 after and before photostability test, controls were covered with aluminium foil and exposed to photostsbility test with Lot 1, 2, 3.

	Lot 1	Lot 2	Lot 3	Control 1	Control 2	Control 3
After						
IC 50 ± SEM	37.88 ±0.28	33.92 ±0.81	34.83 ±1.09	17.35 ±0.16	17.47 ±0.32	18.65 ±0.18
Before						
IC 50± SEM	17.03 ±1.33	17.72 ±1.06	17.94 ±1.11			

UV irradiation samples were tested on organoleptic characteristics. Changes in colour and odour were also observed (**Table 14**).

The controls, which were protected from the light exposure with aluminium foil, did not present changes in both organoleptic features and antioxidant activity.

Table 14: Subjective evaluation of the samples after UV irradiation: O (no change), + (change), ++ (significant change)

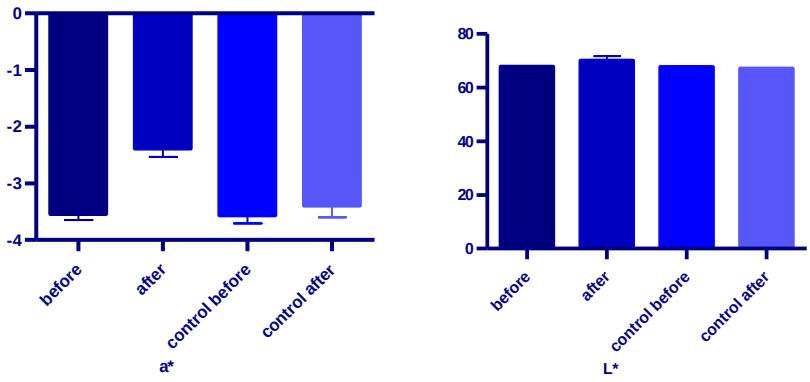
	Colour	Odour
Lot 1,2,3 after	+	++

photostability test		
Control 1, 2, 3 after photostability test	o	o

Colour measurement

Colour of the formulation with C.sativa leaf extract was related to the colour of the green extract due to the presence of chlorophyll. The colour of the samples was objectively determined with tristimulus Minolta Chromameter by L*, a*, b* parameters, which evaluate the colour brightness (L*), changes along a red/green axis (a*), yellow and blue surface (b*).

According to these parameters, **Figure 9** shows evaluation of the colour of the formulation. The samples before and after photostability test were measured. Results are compared in **Figure 13**.



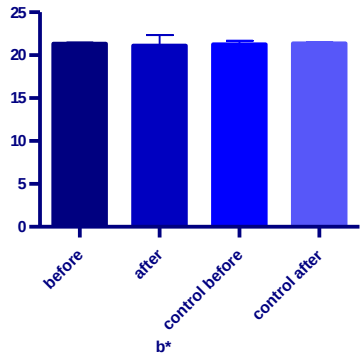


Figure 13: Comparison of the parameters of colour L*, a*, b* for Lot 1, 2, 3 (means±SD) and their controls after and before photostability test

The **Figure 13** shows the differences as means±SD of the samples exposed to the simulated solar irradiation and their controls, which were exposed to the same condition but covered with aluminium foil, which means that controls were protected from simulated solar irradiation. Mainly in graph for parameter a*, we can clearly see the difference before and after photostability test. This parameter represents changes along a red/green axis; it proves the change in the colour of the formulation.

5 CONCLUSIONS

The studied C. sativa leaf extract was shown to have a high antioxidant activity and could be incorporated in topical formulation aiming at protection of skin against damage caused by reactive oxygen species.

Both the carbomer concentration and pH influenced the textural properties of the gel formulations. An increment in the textural parameters was found when the carbomer concentration increased from 0.5 to 2 wt % that is a result of higher number of physical entanglements.

Titanium dioxide concentration did not influence the textural properties and pH of the semisolid formulations.

The physical stability of the gel formulation with incorporated C. sativa leaf extract was shown to be stable in all performed stability tests, except photostability test. This instability could be solved by suitable light resistant package.

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