Reversing the effect of skin aging using *Elegia tectorum* (L.F) Moline & H.P. Linder

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Abstract

All living organisms undergo a process known as aging. During aging, the skin is one of the most visible tissues that is affected. Skin aging is associated with the degradation of the extracellular matrix (ECM) through increased activity of enzymes such as elastase, collagenase and hyaluronidase. In turn the levels of elastin, collagen and hyaluronic acid decrease, resulting in a loss of flexibility and strength of the skin, and hence the formation of wrinkles. The focus of this project was to investigate the inhibitory potential of a South African wetland plant, *Elegia tectorum* (L.F) Moline & H.P. Linder against hyaluronic acid depolymerization by KIAA1199 protein. Hyaluronidase enzymes are able to depolymerize hyaluronic acid into high molecular weight fragments which act as an anti-inflammatory and reduces the formation of scars. If hyaluronic acid is degraded into a low molecular weight fragments, it improves the synthesis of type I and VIII collagen which are structural ECM molecules. The KIAA1199 protein depolymerizes hyaluronic acid independent from other hyaluronidases or the CD44 receptor, the resulting fragments do not have the anti-inflammatory effect, scar reduction effect or collagen synthesis effect as in the case with normal hyaluronidase enzymes, instead wrinkling and sagging of the skin has been observed. This is reason why the KIAA1199 protein should be inhibited. The anti-elastase potential of the plant extract was further determined. Bio-assay guided fractionation was performed in order to identify a bioactive compound with anti-elastase inhibition. Molecular docking studies were performed to predict the binding mode of compounds present in *E. tectorum* to the elastase enzyme. The mutagenic potential and heavy metal content of the plant extract was determined. Furthermore, the stability and *in vivo* anti-wrinkle activity of a formulation containing *E. tectorum* was conducted.

Ethanol, hexane, dichloromethane, ethyl acetate, water, acetone and methanol extracts were prepared from the aerial part of *E. tectorum* and investigated for anti-elastase activity. The ethanolic and methanolic extracts showed the highest inhibition with a fifty percent inhibition (IC_{50}) of 13.50 ± 1.53 and 10.93 ± 4.98 µg/ml respectively, whereas the hexane, dichloromethane, ethyl acetate, water and acetone extracts showed IC_{50} values of 67.47 ± 3.18, 66.12 ± 4.97, 86.20 ± 0.35, 51.14 ± 11.95, 21.80 ± 2.86 µg/ml respectively. This was compared to the positive control, ursolic acid, with an IC_{50} of 8.04 ± 2.82 µg/ml. No significant difference was observed between the ethanolic and methanolic extracts and therefore, the ethanolic extract,

which is a suitable solvent in product development, was selected for further testing. The extract was found to be a competitive inhibitor after performing enzyme kinetic studies, using the elastase enzyme.

In vitro cytotoxicity was investigated on human colorectal adenocarcinoma cell line (HT29) using the Presto-Blue viability reagent. HT29 cells were selected because the KIAA1199 protein was found to be highly expressed by these cells. The extract was found to be non-toxic at the highest tested concentration with an IC_{50} value >400 µg/ml. KIAA1199 protein inhibition was conducted and a one-way analysis of variance (ANOVA) was used to analyze the significance. *Elegia tectorum* was tested at non-toxic concentrations (15, 60, and 240 µg/ml) on the HT-29 cells for 24h, where after the supernatant was collected. The levels of the KIAA1199 protein was analyzed using the Human Cemip/KIAA1199 Elisa kit and compared with the non-treated cells. At the concentrations of 60 and 240 µg/ml the KIAA1199 protein was significantly inhibited when compared with the non-treated cells

The mutagenic potential of *E. tectorum* was determined using the Ames test with *Salmonella typhimurium* TA98 strain in order to identify safety of the extract. The extract was tested at three different concentrations of 5000, 500 and 50 µg/ml and was compared with the positive control 4-nitroquinoline-1-oxide (4-NQO), tested at a concentration of 2 µg/ml, sterile distilled water served as the negative control. At a concentration of 5000, 500, 50 µg/ml of the extract, the number of revertant colonies of *Salmonella typhimurium* strain TA98 was found to be 131.33 ± 10.84, 121.33 ± 26.71, 103.50 ± 14.50, respectively, whereas on exposure to the positive control and negative control revertant colonies were found to be 463.33 ± 40.53 and 100 ± 16.87 respectively. For an extract to be considered mutagenic, it should exhibit a dose-dependent increase in the number of revertant colonies. Additionally, the number of revertant colonies of the extract should be greater than two times that of the negative control. Therefore, the ethanol extract was found to be not mutagenic when compared to the positive and negative control.

Gas chromatography-mass spectroscopic analysis of the ethanolic extract revealed that 57 compounds were present in the extract. Molecular docking of these identified compounds was conducted to predict the binding ability and binding mode to the active site of the elastase enzyme. From the docking scores, five compounds were found to have the closest docking scores as compared to the positive control which was the reference ligand that was docked with the

enzyme. These were: octadecanoic acid with a docking score of -6.92; 9,12,15-octadecatrienoic acid, (Z,Z,Z)- with a score of -6.39; n-hexadecanoic acid with a score of -6.20; 3-(5-methylfuryl)-N-furamidopropionamide with a score of -5.21; bis(2-ethylhexyl) phthalate with a score of -5.02 and hexanedioic acid, bis(2-ethylhexyl) ester with a score of -5.02. The reference compound had a docking score of -11.64 and had a root mean square deviation (RMSD) of 1.68. The lower the docking score, the better the compound fits into the protein.

In order to purify the bioactive compounds; the ethanolic extract was partitioned into ethyl-acetate, *n*-butanol and water fractions. The water fraction showed the highest anti-elastase activity with an IC_{50} of 34.44 µg/ml and therefore; was subjected to column chromatography for further purification. A total of 265 fractions were collected and pooled together into 6 major fractions based on the TLC profile. Most of the fractions had one major compound (confirmed using TLC) which was purified by precipitation. The 6 pooled fractions were tested against elastase enzyme, however no elastase inhibition was noted at the highest tested concentration 250 µg/ml. This could potentially be attributed to the synergistic activity of the compounds in the crude extract as compared to when tested alone.

A formulation was prepared containing 10% v/w of the extract (final concentration of 600 µg/ml). Stability testing of the formulation and extract was conducted to determine their physical integrity and shelf-life at varying temperatures (4, 25, 40 and 50ºC). Accelerated stability testing was conducted over a period of three months where the odor, colour, pH, viscosity and TLC profile was evaluated at each of the temperatures. The results indicated that the product should be stored at temperatures below 30ºC and away from sunlight to ensure a shelf life of 2 years. Additionally, no heavy metal toxicity of the ethanolic extract was detected.

An irritancy test was conducted on 20 healthy volunteers to see if the extract resulted in an adverse reaction on the skin. The extract, when applied neat, was found to be a mild irritant but in aqueous cream (10%), it was found to be non-irritant. During efficacy study, it was found that at 10% of extract in aqueous cream; was effective in decreasing the wrinkles from fourteen 14 (D14) up to twenty-eight (D28) of consecutive use (twice a day) when compared to a placebo control. The study therefore, resulted in a valuable prototype for ageing problems.

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General introduction

CHAPTER 1

1. General introduction

PROBLEM STATEMENT

Aging is a process that is progressive and unavoidable (Robert et al., 1998), however, various factors such UV radiation accelerates skin aging. Aging is caused by the degradation of epidermal cells and proteins, affecting the skins ability to regenerate. This reduces thickness, causes loss of hydration and elasticity ultimately contributing to the formation of wrinkles. Elastin, hyaluronic acid (HA) and collagen which are responsible for skin elasticity, moisture and firmness respectively are part of the extracellular matrix and a reduction in these causes skin aging, (Philips et al., 2010). Aging results in the degradation of elastin by elastase and depolymerization of HA by the KIAA1199. In this study, *Elegia tectorum* a South African wetland plant was tested to determine whether it can be able to inhibit the activity of the KIAA1199 protein which degrades HA, resulting in loss of skin hydration. Bio-assay guided fractionation, to identify the bioactive compounds present within the extract responsible for anti-elastase activity, was performed. Molecular docking studies were performed to predict the binding mode of compounds present in *E. tectorum* to the elastase enzyme. The mutagenic potential and heavy metal content of the plant extract was performed. Furthermore, the stability and in vivo anti-wrinkle activity of a formulation containing *E. tectorum* was conducted. This study gave an insight about a South African wetland plant and its ability to reduce the appearance of wrinkles.

HYPOTHESIS

A South African wetland plant *Elegia tectorum* will reduce wrinkle formation and retain skin hydration.

RESEARCH QUESTION

Can a South African wetland plant and its isolated compounds reduce wrinkle formation and retain skin hydration?

OBJECTIVES OF THE STUDY

1. To make *Elegia tectorum* extracts from different solvents such as ethanol, hexane, dichloromethane, ethyl acetate, water, acetone and methanol
2. To investigate the anti-elastase activity of *Elegia tectorum* extracts using the elastase inhibition assay.
3. To determine the type of inhibition using enzyme kinetics.
4. To determine the cytotoxicity of the most significant extract with anti-elastase inhibition against human colon cancer (HT-29) cells.
5. Determine the inhibitory potential of the lead extract against the KIAA1199 protein.
6. To investigate the anti-mutagenic potential of the lead extract using the Ames test.
7. Identification of compounds present in the lead extract using GC-MS analysis.
8. Predict the binding ability and binding mode of the compounds, identified using GC-MS analysis, to the active site of the elastase enzyme.
9. Bio-assay guided fractionation of the lead extract to identify the bioactive compounds responsible for elastase inhibition.
10. To evaluate the stability of the extract and formulation containing extract.
11. Determine the *in vivo* irritancy and *in vivo* anti-wrinkle activity of the formulation containing the extract.

METHODOLOGY (rationale and motivation)

Elastase inhibition assay

The connective tissue consists of elastic fibers where elastin is found together with other components such as collagen. Elastase degrades elastin, collagen structural and supportive

components by detaching the connective tissue with extensive intracellular fiber networks. By inhibiting the effect of elastase on the structural and supportive components in the dermis, the effect of aging will be minimized as the integrity of these components will be restored. Elastase inhibition assay was used to test the potential of the wetland plant extract to inhibit the elastase enzyme and ursolic acid was used as the positive control as described by Bieth et al., 1978, with slight modifications. The highest concentration of the extract tested was 250 µg/ml and IC_{50} values were obtained. The principle of the assay was that the substrate binds to the enzyme and a product forms, in the presence of an inhibitor such as a plant extract, a product does not form. Enzyme kinetics was used to distinguish between competitive and non-competitive inhibition of the plant extract.

Cytotoxicity

Cytotoxicity on HT-29 cell line was done prior to KIAA1199 protein inhibition assay to make sure that the results of the KIAA1199 protein inhibition are not biased based on the toxicity of the plant extract (it would appear as false inhibition if the cells were killed by the plant extract as they would not produce the KIAA1199 protein). These cells were selected based on that they the express the KIAA1199 protein the most. *In vitro* toxicity was investigated using the Presto-Blue viability reagent. The highest concentration tested was 400 µg/ml and actinomycin D was used as a positive control. It was based on the principle of treating cells with the plant extract, colour changing reagent (prestroblue) was added and it served as an indication of the viability of the cells. This conversion only occurred in viable cells (Lall et al., 2013)

KIAA1199 protein inhibition assay

The process of skin aging is linked a with lack of moisture. One of the major molecules that is responsible for skin moisture is hyaluronic acid (HA) or hyaluronan. Hyaluronic acid is a glycosaminoglycan (GAG) that has the unique ability to bind and retain water molecules in the skin. This molecule is degraded by the protein KIAA1199 which has a major role in the binding and depolymerisation of HA. By inhibiting KIAA1199 protein, this can improve skin moisture

and reduce wrinkle formation. The Human CEMIP/KIAA1199 Elisa kit was used to determine the ability of the lead plant extract to inhibit the KIAA1199 protein. Three concentrations of the extract were tested which were 15, 60, 240 µg/ml on HT-29 cells compared to non-treated cells. The assay is based on the sandwich ELISA principle, each well of the plate was precoated with a target specific capture antibody. The standards as well as the plant extract were added to the wells and the target antigen bound to the capture antibody. Plant extract and standard that was not bound was washed away. A biotin-conjugated detection antibody was added which bound to the captured antigen. Unbound detection antibody was washed away. An Avidin-Horseradish Peroxidase (HRP) conjugate was added which bound to the biotin. Unbound Avidin-HRP conjugate was washed away. A TMB substrate was added which reacted with the HRP enzyme resulting in a colour change. A stop solution which was sulfuric acid was added to terminate colour development reaction and the optical density (OD) of the wells was measured at a wavelength of 450 nm ± 2 nm. The OD of the plant extract was compared to an OD standard curve generated using the known antigen concentrations (LifeSpan Bioscience,Inc., 2019).

Anti-mutagenic assay

Research shows that a lot of plants which are used as food ingredients or in traditional medicine have *in vitro* mutagenic or toxic and carcinogenic properties. Therefore, it is important to screen medicinal plants for their mutagenic potential. Mutations are the cause of innate metabolic defects in cellular systems, triggering morbidity and mortality in living organisms. The Ames test was used to determine the antimutagenicity potential of the plant extract. Three concentrations of the plant extract were tested which were 5000, 500, and50µg/ml. the positive control was 4-nitroquinoline-1-oxide (4-NQO), tested at 2 µg/ml. The Ames test was based on the principle of using *Salmonella typhimurium* tester strains TA98 to test whether the plant extract has the potential to cause mutations. Point mutations were made in the histidine operon, this made the bacteria not to be able to produce histidine. The mutation resulted in his- organisms that could not grow unless histidine was present. Culturing his- *Salmonella* in media with plant extract and other chemicals could result in mutations in histidine encoding gene, which could regain the ability to produce histidine (his+). These reverted bacteria could grow in

media that is histidine deficient (Maron and Ames 1983, modified by Mortelmans and Zeiger 2000).

Molecular docking

Molecular docking was used to predict molecular recognition structurally, binding modes, binding affinity and binding free energy of the compounds found within the extract to the elastase enzyme. Gas chromatography–mass spectrometry (GC-MS) of the ethanolic extract of *E.tectorum* was conducted and 57 compounds were identified. Structure selection and protein preparation was conducted using Schrödinger's Protein Prepreration Wizard. Hydrogen bonds were optomised using PROPKA at a pH of 7.0. minimization of the complex was conducted using OPLS force field (Harder et al., 2016). In order to confirm the target sites on the crystal structure of the elastase enzyme, SiteMap was used (Schrödinger, LLC, NewYork, NY, 2018; Halgren, 2007, 2009). Glide was used for docking the ligand (*E.tectorum* compounds) to the elastase enzyme site in order to estimate the binding affinities (Halgren et al., 2004) .

Compound isolation

Isolation is essential as it gives an indication of the different kinds of compounds that are present in the plant responsible wrinkle reduction. Silica column chromatography was used to isolate different compounds. The stationary phase was a solid adsorbent which was put in a column. The mobile phase was a liquid that was added at the top and passed through the column using gravity. The plant extract of *E.tectorum* was added inside at the top of the column followed by a liquid solvent which also passes through the column by gravity. Different compounds in the extract have different interactions with the stationary and mobile phase, these were carried along the mobile phase at different degrees and separation was achieved. The individual elutants (fractions) were collected as the solvent dripped from the bottom of the column (Organic Chemistry at CU Boulder, 2020). Elastase inhibition assay was used to screen the isolated fractions and Nuclear Magnetic Resonance (NMR) was used to analyse the compound that was present in one of the fractions.

Stability testing

Stability testing was completed to indicate how stable is the extract as well as the formulation with the plant extract in a period of three months at four different temperatures. It is important to understand the effect of storage, shipping conditions on the shelf life of a product. Major concerns are sunlight exposure, temperature, vibration and humidity. This is the reason behind formulations being stored under regulated testing conditions and analyzed at fixed intervals (Particle Science, 2011). The plant extract and formulation with extract were placed at four different temperatures 4ºC, 25 ºC, 40 ºC and 50 ºC. Odour, pH, appearance and viscosity are parameters that were investigated over a period of 12 weeks.

Irritancy and efficacy studies

An irritancy test was also conducted to see if the plant extract resulted in an adverse reaction on the skin. When in contact with the skin, some plants can cause irritant, allergic and phototoxic dermatitis (Davis et al., 2011). Twenty (20) subjects between the ages of 21 and 64 were chosen and the plant extract was applied on their forearm. The forearms were analysed after 24, 48 and 72 hours. A visual rating system was used to identify the reactions: 0 = no response, 0.5 = minimal/doubtful response, 1 = mild erythema, spotty or diffuse, 2 = definite erythema, uniform redness, itching or burning response and swelling may occur, 3 = strong and severe uniform redness, swelling or spreading beyond the area of the disc may occur, and 4 = fiery redness, oedema, papules, bullae. An efficacy study was conducted by the industry, “Future Cosmetics”. Details are in Appendix C.

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Chapter 2

Literature review

CHAPTER 2

2.1. The skin

The skin is defined as the layer of tissue that is used as an outer cover of the body of a person or an animal. It is important because it protects the body against severe temperature fluctuations, dangerous chemicals, ultraviolet radiation from the sunlight and water loss. It furthermore acts as a barrier against bacterial infections. It uses vitamin D from the sun, which helps the body to absorb calcium, main building blocks of bones. The skin is usually soft and elastic when we are born, and as we grow older, facial skin starts to wrinkle due to an inability to maintain moisture and elasticity (National Geographic, 2017). The skin is made up of three main layers; the epidermis, dermis and hypodermis (subcutaneous layer) (Figure 2.1.1) (Rittie and Fisher, 2002).

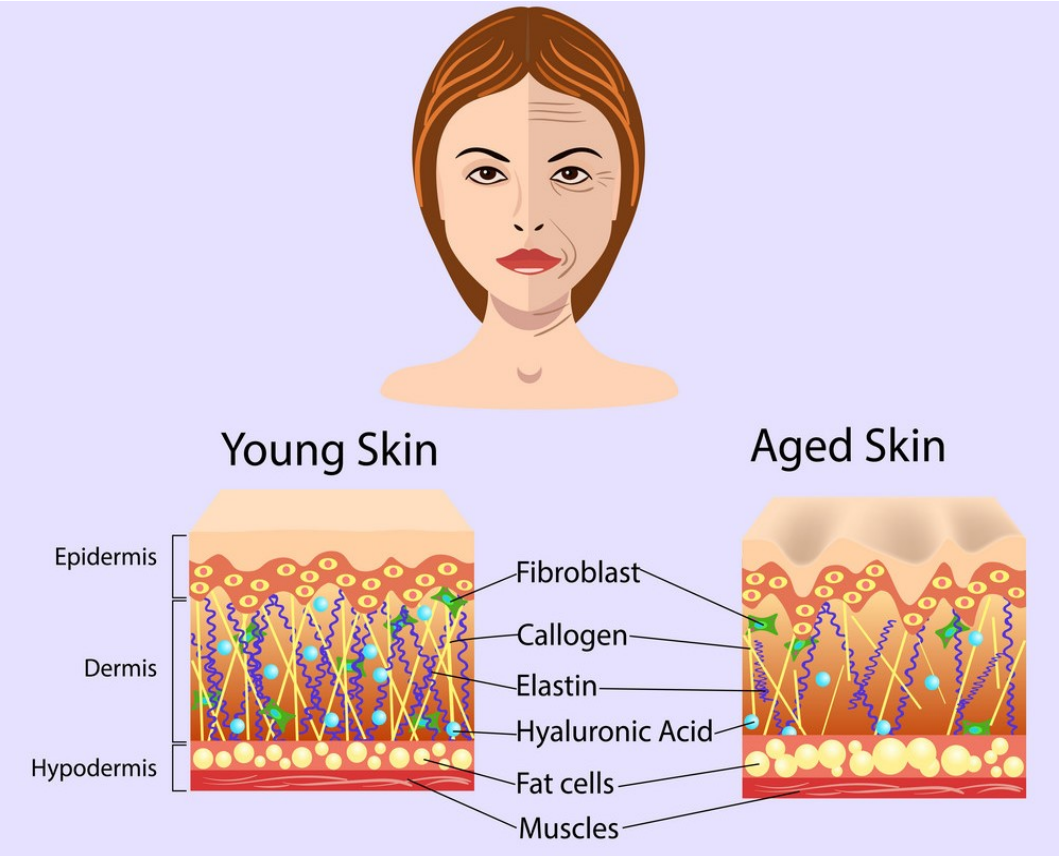


Figure 2.1.1. Three different layers of the skin, the epidermis, dermis, hypodermis and the components that make up the layers. Collagen, elastin and hyaluronic acid are degraded in aged skin compared to young skin which results in wrinkle formation (Vector Stock, 2020).

These layers differ in structure between younger and older people.

The epidermis is composed of numerous intersecting layers of skin cells. The outer part is made up of cells that are flat, dry and high in keratin. Keratin is a protein that is found in nails and hair, its function is to maintain moisture inside the skin. New skin cells are produced in the lower epidermis, where they are processed (Rittie and Fisher, 2002; National geographic, 2017).

The dermis layer is situated under the epidermis, it is thick, strong and elastic. In the dermal extracellular matrix there are certain components that are responsible for strengthening of the skin such as elastin, collagen type I and III, fibronectin, proteoglycans and collagen fibrils. During photo-aging, the skin experiences changes in the dermal connective tissue, changes in the connective tissue with regards to quantity and structure contributes to wrinkle formation. It is having a network of small blood vessels that function in controlling body temperature (Puizina-Ivic, 2008).

The hypodermis (subcutaneous layer) is found below the dermis. It comprises of subcutaneous fat and adipocytes which functions as a fuel reserve during food shortage and as an insulator for internal organs protecting an individual from trauma such as falls and knocks (National geographic, 2017). It is also made up of fibrous bands which anchor the skin to the deep facial collagen and elastin fibers that join the subcutaneous tissue to the dermis, lymphatic vessels which to link the dermis to the lymphatic system. It contains nerves that connect the dermis and free nerve endings to the nervous system as well as blood vessels that connect the dermis to the circulatory system (News Medical Life Sciences, 2019).

Collagen which makes up 80% of the skin's dry weight strengthens the skin, whereas elastin which makes up 2-3% of the ECM is responsible for skin elasticity. Glycosaminoglycans (GAG's) are responsible for the hydration of the skin. Each of these components are formed by fibroblast cells and in most cases are affected by photo-aging (Thring et al., 2009).

2.2. The mechanism of aging

Aging skin is a common dermatologic concern in people; this may be due to the obsession of looking younger. In order to improve the appearance of the skin, individuals are continuously

looking for creams and procedures that will help them look younger. In 2004, United State retail sales accounted for over $12.4 billion on cosmetics and cosmeceuticals which are products that have medicinal properties. In 2010, over $16.5 billion in sales was predicted from the anti-aging market (Helfrich et al., 2008). Like all organs, the skin suffers progressive physiological and morphological deterioration with time, this is due to various cumulative factors which are intrinsic and extrinsic. Aging skin is marked with decrease in elasticity, the skin can be dry, flaky and rough, with fine and coarse wrinkles as well as impaired wound healing (Vayalil et al., 2004; Brenneisen et al., 20020). There are two types of skin aging, namely: chronological aging (intrinsic) and photo-aging (extrinsic).

2.2.1. Intrinsic aging (chronological aging)

Intrinsic aging happens naturally due to alterations in the structural support of the bones in the face and alterations in the arrangement of muscles. Intrinsic aging is characterized by a continuous accumulation of reactive oxygen species (ROS), as a result of oxidative cellular mechanisms. Reactive oxygen species cause damage to the cellular constituents such as enzymes, DNA and,-membranes and reduce the level of hormones that affect sexual development for example estrogen and testosterone (Puizina-Ivic, 2008).

Telomeres, which are located at each end of a chromosome, also play a role in intrinsic aging. During each cell division, telomeres become shorter and it is estimated that during childhood 30% of telomere length is lost. The telomerase enzyme helps to maintain this telomere sequence TTAGGG and keeps it from shortening. During cell division, the shortening of the telomere sequence occurs due to the fact that the ends of the chromosomes are not protected. The cell then reaches senescence, where it cannot proliferate but it is still viable (Puizina-Ivic, 2008).

Sex hormones play a major role in aging as they decrease with increasing age. Sex steroids that are known to decrease are estrogen, testosterone and dehydroepiandrosterone (DHEA). Growth hormones also tend to decrease with increasing age as well as other hormones such as melatonin, insulin, thyroxine and cortisol. Levels of certain signaling molecules such cytokines and chemokines have also been shown to increase with aging, leading to the damage of the skin, such as the transforming growth factor beta1 (TGF-β1)which results in fibroblast senescence (Puizina-Ivic, 2008). Tumor necrosis factor α (TNF-α) has a major role in proinflammatory

process as it inhibits collagen synthesis and induce the production of MMP-9, a type IV collagenase. Collagenase activity in fibroblast cells is induced by high concentrations of TNF-α, resulting in a decrease in collagen production. Some age-related processes such as skin inflammation is promoted by interleukin (IL)-1 and IL-18 which increase with age. Formation of wrinkles is also associated with an increase in proinflammatory cytokine, IL-6, after menopause (Kim and Park, 2016)

In the epidermis there are not many changes in the stratum corneum, thickness of the epidermis, and keratinocyte shape, but there is a reduction in the number of melanocytes and Langerhans cells (Figure 2.2.1). Many changes are observed at the dermal-epidermal junction where, the rete ridges become flat therefore decreasing the surface contact of the dermis and epidermis resulting in a decrease in exchange of nutrients and metabolites between the dermis and epidermis, a loss in dermis volume and a reduction in blood supply due to decreased number of vessels. (Puizina-Ivic, 2008). The number of fibroblasts in the skin reduce as well as collagen and elastin levels. The association of elastin and collagen fibers is damaged due to the decrease in protein synthesis, which affects type 1 and 3 collagens in the dermis. There is also an increase in the degradation of proteins found in the ECM (Rittie and Fisher, 2002).

Skin undergoing chronological aging looks dry, thin, unblemished and exhibits little elasticity (Rittie and Fisher, 2002). There are sequential changes that happen to follow a basic progressive pattern through time; therefore, the rate of aging is different amongst individuals.

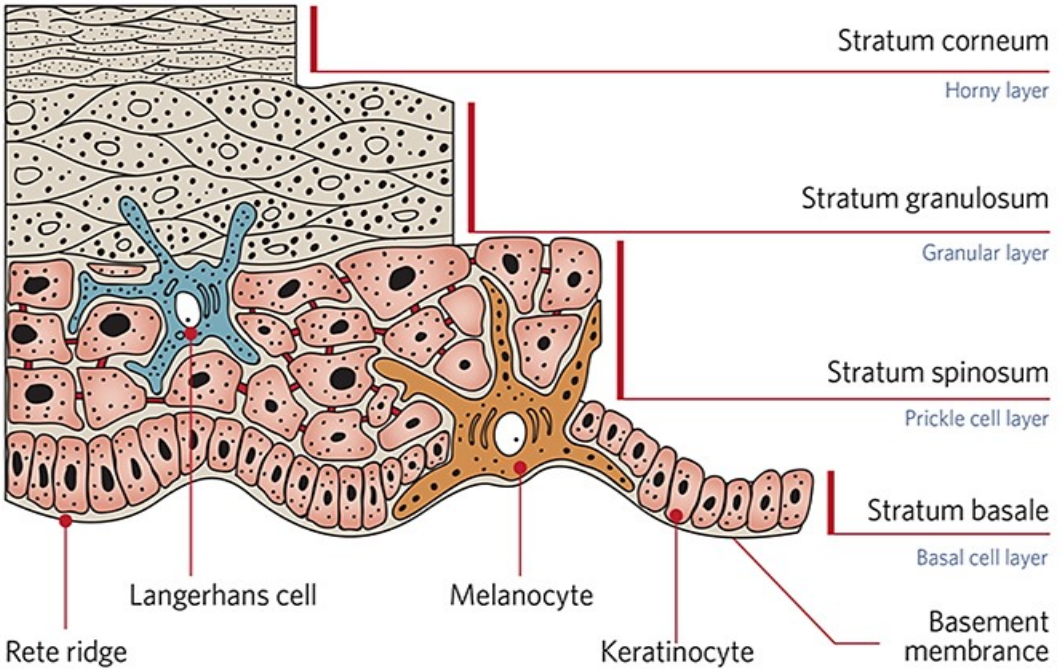


Figure 2.2.1. The anatomy of the epidermis, the stratum corneum is made up of highly keratinized cells which are flattened, these cells are lined with keratins and are shed from the epidermis as dead skin cells. The stratum granulosum consists of flattened and large polygonal granulated cells which have nuclei, these cells undergo final cell differentiation of the uppermost keratinocyte of the stratum spinosum. The thickest layer of the epidermis is the stratum spinosum, it is made up of polyhedral-shaped keratinocytes and Langerhans cells are found in this layer. Langerhans cells are derived from monocytes of the bone marrow and initiate an immune response against foreign substances. Stratum basale consists of a single layer of basophilic column shaped keratinocytes which have a large nucleus. Melanocytes are cells that produce melanin in the skin, and they are found amongst the cells of the stratum basale (Baker, 2016).

2.2.2. Extrinsic aging (photo-aging)

Extrinsic aging is triggered by environmental factors such as UV radiation, harsh weather, pollution, cigarette and smoke, physical and psychological stress, alcohol intake, lack of sleep and poor nutrient intake. These factors affect the integrity of elastin and collagen which are the structural proteins responsible for the elasticity of the skin (Albert et al., 2007). The major contributing factor to extrinsic aging is exposure to UV radiation, contributing 80% to this type

of aging (Puizina-Ivic, 2008). There are three types of UV radiation, UVC (100-290nm), UVB (290-320nm) and UVA (320-400nm) (Puizina-Ivic, 2008).

UVC is mostly blocked by the ozone layer and therefore does not reach the Earth's surface and has no impact on the skin (Farage et al., 2008). UVB is known to cause burning of the skin and causes changes at the epidermal level; this is where majority of UVB is absorbed. It disrupts the DNA in keratinocytes and melanocytes and induces the assembly of soluble epidermal factors (ESF) and proteolytic enzymes, which are present in the dermis after UV exposure. UVB exposure results in the occurrence of thymidine dimers, which is the formation of strong covalent bonds between two thymidines. These bonds disrupt transcription and replication, and if not repaired can lead to mutations. Exposure to UV radiation can lead to the development of carcinomas, actinic keratosis, melanomas and lentigines (Puizina-Ivic, 2008).

UVA, known to cause photo-aging, has the ability to penetrate further into the skin, reaching the dermis layer, thereby causing disruption to both the dermis and epidermis. Synthesis of collagen degrading enzymes, xeroderma pigmentosum factor (XPF) (also found in the epidermis) and matrix metalloproteinases (MMPs) is increased by UV radiation. Xeroderma pigmentosum factor causes epidermal-dermal folding, indicating the beginning of wrinkle formation. Type IV and VII collagen is present in lower amounts at the base of wrinkles, since there is less, wrinkles become deep. Different MMPs degrade different dermal matrix proteins; for example, MMP-1 degrades collagen I, II, III. The proteolysis can occur due to the imbalance between activation and inhibition of enzymes. UVA can further produce ROS which are able to cause DNA strand breaks and affect lipid peroxidation (Puizina-Ivic, 2008).

Pollutants such as long-term smoking further contributes towards skin aging. The skin becomes yellow and irregularly thickened due to the breakdown of elastic tissue. Both sun exposure and smoking can induce the production of MMPs. Facial stratum corneum moisture is decreased by smoking as well as the level of vitamin A, which its function is to prevent collagen damage. Other pollutants such as nitrogen oxide, volatile organic compounds resulting from burning of fossil fuel and emissions from factories cause the skin to look wrinkled, coarse, exhibit age spots and uneven pigmentation (Puizina-Ivic, 2008).

Intrinsic and extrinsic aging occur due to different processes, however these two processes do share similar molecular pathways such as the production of ROS (Papakonstantinou et al., 2012; Avantaggiato et al., 2015).

2.3. Elastase

Elastase is an enzyme belonging to the chymotrypsin family of proteases. It is responsible for the degradation of the structural components of the dermis, such as elastin. Elastase can cleave elastin, collagen and other proteins found in the ECM. After cleavage of elastin, elastase causes wrinkle formation and the skin starts to sag (Thring et al., 2009). An increase in elastase activity results in certain diseases such as rheumatoid arthritis, premature skin aging, chronic obstructive airway disease, cystic fibrosis, psoriasis and delayed wound healing (Jabs, 2012). According to Tsuji et al (2001), at least two types of elastases exist in the skin; neutrophil elastase and skin fibroblast elastase. Neutrophil elastase is a serine proteinase, whereas skin fibroblast elastase belongs to the metalloproteinases family. These two elastases vary in terms of substrate specificity. Neutrophil elastase has the ability to degrade all types of elastic fibers and is highly sensitive to elaunin and mature elastic fibers. Skin fibroblast elastase acts on elaunin fibers and oxytalan and, has limited sensitivity to mature elastic fibers. In various types of tissues, these elastases are implicated in the metabolism of elastin fibers during aging, inflammation and diseases. In wrinkles that form due to UV radiation, there is not sufficient information about the exact role of these elastases, although it has been reported that overproduction of elastases induced by UV radiation affects the elastic-fiber network of the skin (Tsuji et al., 2001).

Elastin is the main constituent of elastin fibers, it is a crosslinked protein polymer that is available in the ECM (Sage and Gray, 1979). Elastin has elastic properties, which make it important as it gives the skin, lungs, arteries and ligaments elasticity (Thring et al., 2009). During aging, elastin is degraded by elastase which is followed by loss in elastic fibers (Robert et al., 1983).

2.4. Hyaluronic acid

Hyaluronic acid is widely distributed in the human body. It is abundant in the skin (Tammi et al., 1988; Juhlin, 1997; Armstrong and Bell 2002; Tzellos et al., 2009; Tzellos et al., 2011). It is also found in the umbilical cord (Weissmann and Meyer, 1957), the vitreous of the eye (Meyer and Palmer, 1934), synovial fluid (Hamerman and Schuster, 1958) and in all tissues and fluids of the body for example the skeletal tissues, the lung, heart valves, the prostate, the aorta, corpora cavernosa, tunica albuginea and penis (Papakonstantinou et al., 2012).

More than 50% of the total body HA is contained in the skin (Reed et al., 1988) It is responsible for skin hydration and protects the skin against free radical damage, particularly against UVA and UVB (Meyer and Stern, 1994). Solar UV radiation of the skin can induce sunburn and transient inflammation that can result in premature skin aging (photo-aging) and skin cancer (Tsoureli et al., 2006; Dai et al., 2007; Nichols and Katiyar, 2010). Damage to skin functions, triggered by UV, creates a cosmetic as well as medical problem affecting a large portion of the population. Therefore, the effective development of substances that could potentially treat or prevent UV damage to the skin are essential (Nichols and Katiyar, 2010).

Hyaluronic acid is a non-sulphated GAG that is made up of repeating polymeric disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine which are linked by glucuronidic β (1→3) bond (Figure 2.3 A) (Wiessmann and Meyer, 1954; Wiessmann et al., 1954). Hyaluronic acid forms specific stable tertiary structures in aqueous solution (Figure 2.3 B) (Scott and Heatley,1999). Regardless of its simple composition, without variations in its sugar composition or without branching points, HA has a variety of physiochemical properties. They appear in various shapes and configurations depending on their sizes, concentration, associated cations and pH (Laurent, 1970). It is not covalently attached to a protein core like other GAG molecules, but it can form aggregates with proteoglycans (Bates, 1984). Hyaluronic acid gives solutions high viscosity, even at low concentrations as it encompasses a large volume of water (Turino and Cantor, 2003).

The biological roles of HA depend on the size of the chain length, molecular mass and on the circumstances under which they are synthesized (Noble, 2002; Toole, 2004). Extracellular high

molecular weight HA fragments (HMWHA) inhibits endothelial cell growth and is thus anti-angiogenic in nature. These polymers also have an increased ability to bind fibrinogen; this is one of the first and major reactions to form in clot formation, which are important in early wound healing (Chen and Abatangelo, 1999). Such HA polymers have anti-inflammatory and immunosuppressive activity. High molecular weight HA fragments are highly concentrated in fetal circulation and amniotic fluid, this indicates that it is responsible for immunosuppression in the developing fetus (Grish and Kemparaju, 2007). Furthermore, HMWHA is involved in inhibiting the formation of scars (Noble, 2002).

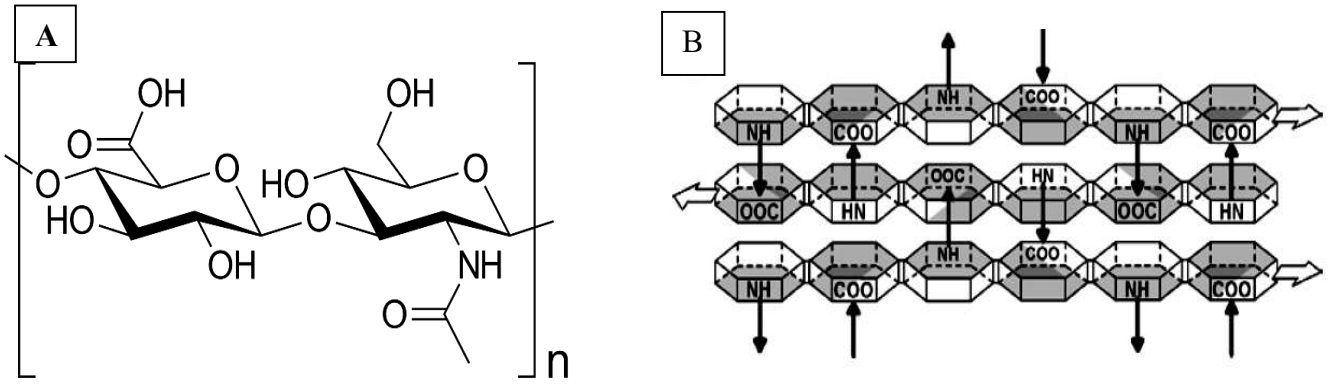


Figure 2.4.1. (A) Chemical structure of HA, (Vaccinationist 2016). (B) Schematic illustration of the tertiary structure arrangement of HA in aqueous solution. HA molecules may bind to each other in a repeated antiparallel array, with hydrophobic patches (shaded surfaces) on the faces of the rings (hexagons) stacking against each other, and intermolecular hydrogen bonds between amide and carboxylate groups on adjacent molecules (arrows) providing specific stabilization to the network. The amide group is trans in this model and the HA chains are required to adopt an extended 2-fold helical conformation (Blundell et al., 2006).

Lower molecular weight HA (LMWHA) fragments (400-1000 kDA) interact with different types of receptors that activate signaling cascades and cause profound changes in the behavior of cells. This polymer has been proven to promote angiogenesis in several experimental models (West et al., 1985; Noble, 2002; Toole, 2004). It also improves the synthesis of type I and VIII collagen

which are ECM molecules of the endothelial cell angiogenic phenotype. LMWHA are potent stimulators of inflammatory cytokine and adhesion molecules such as IL- 1β, TNFα, and IGF-1 and CD44 (Noble et al., 1993).

Shorter HA (100-400 kDA) fragments have been reported to promote cell proliferation of chondrocytes, endothelial cells and fibroblasts (Papakonstantinou et al., 2012). Iacob and Krudson (2006) reported that HA fragments are essential in the activation of nitric oxide synthase and the production of nitric oxide (NO) by articular chondrocytes. This activation of NO production by HA fragments has been reported in macrophages, endothelial cells, rat liver, Kupffer cells located in the liver functioning as macrophage, breast cancer cells (MCF7), and cervical cancer cells (HeLa). In some cases, the stimulatory effects of HA fragments were shown to be mediated by the activation by the nuclear factor (NF)-κβ (Horton et al., 1999).

Smaller HA oligomers (>100 kDa) are strong activators of dendritic cells, the antigen presenting cells of the immune system. Therefore, HA fragments have the ability to be angiogenic, immune-stimulatory and inflammatory (Noble, 2002; Termeer et al., 2002; Rossler and Hinghofer-Szalkay, 2002; Stern, 2003). Angiogenic HA fragments stimulate endothelial cell proliferation, adhesion and migration by activation of focal adhesion kinase and mitogen activated protein (MAP) kinase pathways (Rossler and Hinghofer-Szalkay, 2002; Murai et al., 2004).

Very small HA (>20 kDA) oligosaccharides are also responsible for specific and unique biological activities. Oligomers that are made up of six disaccharides promote differentiation of endothelial cells induced in response to the angiogenic effect of bigger HA fragments. Furthermore, control of migration, maturation and signaling in skin keratinocytes is also closely associated with small HA oligomers (Takahashi et al., 2005).

Tetra and hexasaccharides are the main products of hyaluronidase-mediated degradation. These induce the expression of heat shock proteins (Hsps), they are also anti-apoptotic meaning they suppress cell death in cultures undergoing hyperthermia (Xu et al., 2002). Another study by Ghatak et al., 2002, showed that the addition of tetrasaccharides inhibits anchorage-independent growth of several tumour cell types by suppressing the phosphor-inositol-3-kinase/ Akt pathway.

The process of skin aging is linked with lack of moisture. Hyaluronic acid is responsible for skin moisture, as it has unique ability to bind and retain water molecules. It is found in the extracellular matrix (ECM). In the past years, the constituents of the skin have been well characterized. Many studies focused on the cells that make up the skin layers for example the subcutaneous layer, the dermis and the epidermis. In recent years, it is acknowledged that ECM molecules that are found between cells offer a constructive framework and have major effects on cellular function. The ECM molecules form a highly organised structure consisting of GAG, proteoglycans, structural proteins such as elastin and collagen as well as growth factors. Hyaluronic acid is the major component of the skin ECM and it is found in both the dermis and epidermis, the dermis has a greater proportion of HA (Papakonstantinou et al., 2012; Erickson and Stern, 2012). Therefore, HA has an important role in anti-aging.

2.4.1. Synthesis of Hyaluronic acid

Hyaluronic acid is synthesized by specific enzymes known as the HA synthase (HAS). These enzymes are membrane bound and synthesize HA on the inner surface of the plasma membrane where after HA is extruded through pore-like structures into the extracellular space (Prehm, 1984; Prehm, 1990). In mammals there are three of these enzymes; HAS -1, -2, and -3 which have different enzymatic properties and synthesize HA chains of different size (Weigel et al., 1997; Itano et al., 1999; Itano et a.l, 2002). These enzymes are regulated differently, HAS-1, HAS-2 HAS-3 genes are up regulated by transforming growth factor beta (TGF-β) in the epidermis and dermis, however there are major differences in the kinetics of the TGF-β response between the two compartments. This suggests that these genes are regulated independently, and HA plays different roles in the dermis and epidermis (Stern and Maibach, 2008). In skin keratinocytes, HAS-2 is induced by growth factors to stimulate HA synthesis. In the epidermis, HAS-3 has a role in synthesizing HA (Choi et al., 2019)

2.4.2. Catabolism of Hyaluronic acid

There are two separate mechanisms that are involved in the breakdown of HA this can be enzymatically, by a class of enzymes known as hyaluronidase or through cleavages that are nonenzymatic oxidation reactions (Erickson and Stern, 2012).

Karl Meyer introduced the term hyaluronidase to denote the enzymes that degrade HA (Meyer, 1971). Hyaluronic acid has a half-life of less than a day in the skin, 3 to 5 minutes in the blood and 1 to 3 weeks in the cartilage (Fraser et al., 1981; Reed et al., 1990; Laurent et al., 1991). It is degraded into fragments of different sizes by hyluronidases (HYAL), this is achieved by hydrolysing the hexosaminidic β (1→4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues in HA. There are six HYAL in humans that have been identified so far; HYAL -1, -2, -3, -4, PH-20 and HYALP1 (Stern and Jedrzejas, 2006). These enzymes received little attention in the past due to that they are found at extremely low concentrations and are difficult to purify, characterize and measure their activity, which is high but unstable (Stern and Maibach, 2008). In serum, HYAL-1 is the major HYAL. Mutations in the HYAL-1 gene are related to HYAL deficiency and mucopolysaccharidosis type IX. Hyaluronidase-2 has lower activity compared to plasma HYAL-1 and it specifically hydrolyses HA of high molecular weight therefore, resulting in HA fragments of approximately 20 kDa. These fragments are further degraded to smaller oligosaccharides PH-20. HYAL-3 found in the bone marrow and testis as well as other organs including the lungs. Its role is not completely understood in the catabolism of HA, it is proposed that it contributes to HA degradation by enhancing the activity of HYAL-1 (Papakonstantinou et al., 2012).

Non-enzymatic degradation of hyaluronic acid can occur, which is caused by free radicals in the presence of reducing agents such as ascorbic acid, ferrous ions, thiols, as well as cuprous ions, a process that needs the presence of molecular oxygen. Therefore, mechanisms or agents that can delay the free-radical-catalyzed degradation of HA can have a major impact in maintaining the integrity of dermal HA and its moisturizing properties.

2.4.3. Effects of inhibiting hyaluronidases (HYAL's)

Hyaluronidase does not only disrupt the ECM, it has various other roles in biological and physiological processes such as embryogenesis, angiogenesis, inflammation, wound healing, disease progression, bacterial pathogenesis, diffusion of systematic venoms and invasion of tumours (Sahasrabudhe and Deodhar, 2010). Hyaluronidase is an endoglycosidase which

cleaves HA in tissues therefore, this increases the membrane permeability, decreases viscosity and renders tissues more permeable to injected fluids (spreading effect). The enzymes can be used therapeutically to increase the speed of absorption, to increase the effectiveness of local anaesthesia, promote the resorption of excess fluids and to diminish tissue destruction by subcutaneous and intramuscular injection of fluids (Frost et al., 1996; Farr et al., 1997). Hyaluronidase has been used to decrease the extent of tissue damage following extravasation of parental nutrition solution, antibiotics, electrolyte infusion, aminophylline, mannitol and chemotherapeutic agents including vinca alkaloids (vincristine, vinblastine, vinorelbine) (Muckenschnabel et al., 1997). They can be used in many fields such as surgery, orthopaedia, ophthalmology, oncology, internal medicine, dermatology and gynaecology (Borrelli et al., 1986; Manzel and Farr, 1988; Frost et al., 1996;). Therefore, this shows that inhibition of hyaluronidases is not a preferred option, rather inhibit the KIAA1199 protein which also degrades HA.

2.4.4. Current treatment for HA loss

Pharmacological agents such as oestrogen, growth factors, retinoic acid and cytokines are used to stimulate HA synthesis and prevent skin atrophy, dryness and wrinkles in aging people (Park et al., 2017). Medicinal plants such as *Arctium lappa* and *Areca catechu* have been reported to have anti-hyaluronidase activity *Radix astragali* has been reported to stimulate hyaluronic acid production (Binic et al., 2013). Black tea (*Camellia sinensis*) was found to have anti-hyaluronidase activity and is used extensively in anti-aging and anti-wrinkles cosmetics. Soyabean (*Glycine max*) is rich in hyaluronic acid, it has been reported to decrease the volume and depth of wrinkles and maintains the hydration of the stratum corneum (Hooda, 2015).

2.5. KIAA1199 Protein

The KIAA1199 gene is found on the 15q25 chromosome and encodes a 150-kDa protein (1361 amino acids). Initially it was described as an inner ear protein in which three point mutations were found to be related with non-syndromic hearing loss (Abe et al., 2003). It is made up of G8 domain, which has eight conserved glycine residues, it consist of five β-strand pairs and one α-

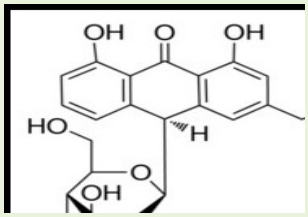
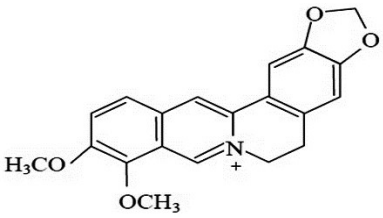
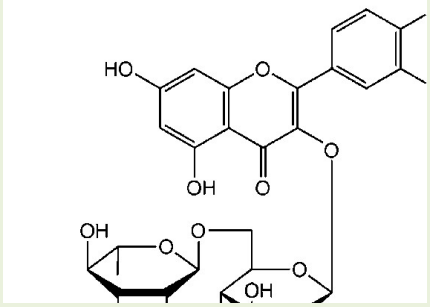
helix, four pbH1 domains, which are made up of parallel β-helix repeats and two GG domains, each having seven β-strands and two α-helices. In recent years, KIAA1199 has been found to play a major role in hyaluronic acid binding and depolymerisation (Yoshida et al., 2013[a]).

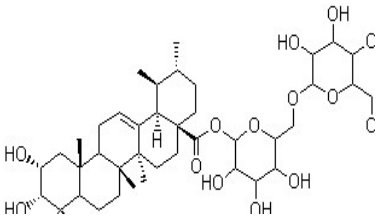
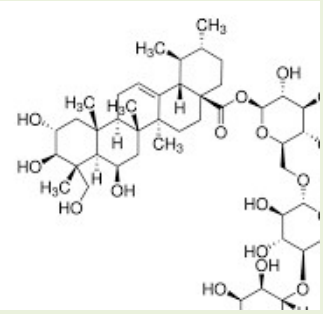
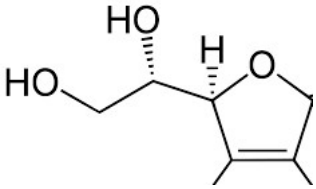
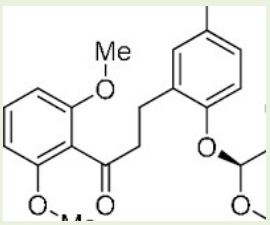
Yoshida and colleagues (2013)[a], conducted a study where they tested the involvement of HYAL 1, HYAL2 and CD44 in HA depolymerization in normal skin fibroblast and found that the knockdown of these genes with siRNAs did not inhibit HA depolymerisation. This caused them to further investigate new mechanisms for HA degradation. This study provided unique evidence that a deafness gene of unknown function, known as the KIAA1199 played a major role in the binding and depolymerisation of HA, without the use of HYAL enzymes and surface HA receptor CD44.. In their study they also showed KIAA1199 is expressed mostly by dermal fibroblast in normal skin and is overexpressed by synovial fibroblasts and tissues from arthritic joints. This expression of KIAA11999 by dermal fibroblasts in normal skin and by synovial cells in synovial tissue suggests that this molecule plays role in catabolising HA in the dermis of healthy skin and synovium of arthritis patients. The study concluded that therapeutic interventions targeting KIAA1199 may be of clinical importance ([a]Yoshida et al., 2013; [b]Yoshida et al., 2013; Yoshida et al., 2014). Therefore, KIAA1199 protein inhibition is a novel mechanism that was targeted in this study.

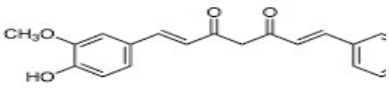
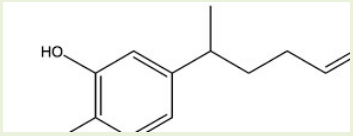
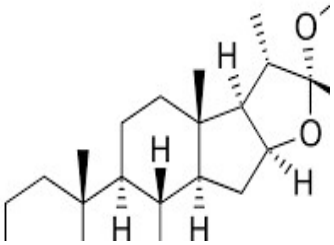
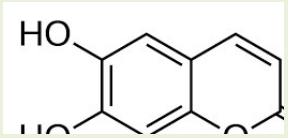
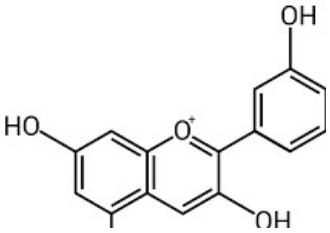
2. 6. Phytoconstituents with anti-aging potential

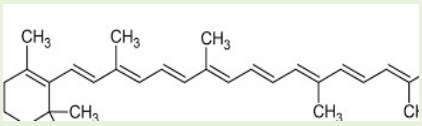
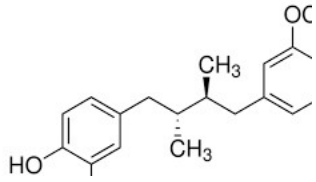
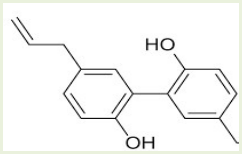
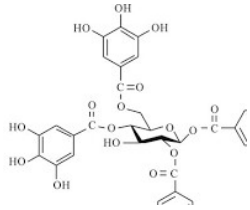
Plants are known to be a potential solution to fight skin aging, since certain plant extracts can defend the skin from aging via the inhibition of enzymatic degradation (in this case inhibition of elastase from degrading elastin). Secondary metabolites such as polyphenols, triterpenoids and tannins are widely investigated and have anti-elastase activity. An example is the polyphenols that are isolated from green tea (*Camellia sinensis*) such as epigallocatechin gallate (EGCG) and catechin, these are found to be inhibitors of elastase and collagen (Thring et al., 2009). Table 2.6.1 shows compounds isolated from plants which have been shown to have wrinkle reduction effect.

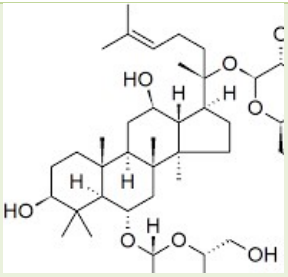
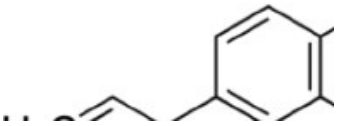
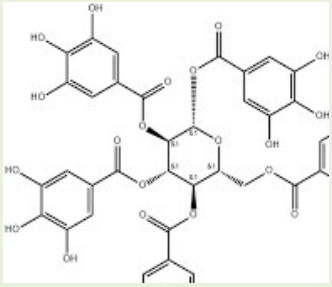
Table 2.6.1. Compounds isolated from plants which have a wrinkle reduction effect.

Compounds	Species	Mechanism	References
Aloin A 	*Aloe barbadensis* Miller	Stimulates the production of collagens and elastin. This results in rejuvenation and reduction of fine lines and wrinkles. It eliminates dead cells and promotes new skin cells	Sharma et al., 2013.
Berberine 	*Aloe vera*	Inhibited basal and TPA-induced expression and activity of MMP-9 Prevents UV-induced MMP-1 and reduction of type I procollagen expression	Kim et al., 2008 Kim and Chung 2008
Rutin 	*Calendula officinalis* L.	Regulates the activity and secretion of MMP-9 and MMP-2	Yris et al., 2010

Asiaticoside 	*Camellia japonica* L.	Induces human collagen I synthesis through TGFβ receptor I kinase	Lee et al., 2006
Madecassoside 	*Centella asiatica*	Induce collagen expression and/or to modulate inflammatory mediators	Haftek et al., 2008
Ascorbic acid 	*Centella asiatica* L. Urban	Improves clinical score for deep and superficial wrinkles, suppleness, firmness, roughness and skin hydration Induce type-I collagen synthesis	Haftek et al., 2008
Curculigoside 	*Curculigo orchioides* G.	Inhibits the expression of MMP-1	Lee et al., 2009

Curcumin 	*Curcuma longa* L	Inhibits the expression of MMP-2	Sumiyoshi and Kimura, 2009
Xanthorrhizol 	*Curcuma xanthorrhiz*a Roxb.	Inhibits the expression of MMP-1	Oh et al., (2009)
Diosgenin 	*Dioscorea composita* syn. *Dioscorea villosa*	Increases the uptake of bromodeoxyuridine and intracellular cAMP levels in keratinocytes	Tada et al., 2009
Esculetin 	*Fraxinus chinensis* Roxb	Decreases the expression of MMP-1 mRNA	Lee et al., 2007
Anthocyanins 	*Glycine max* L. Merr.	Inhibits melanosome phagocytosis and prevents the activation of caspase-3 pathway	Tsoyi et al., 2008

β-carotene. 	*Labisia pumila* (Blume)	Inhibits the expression of TNF-alpha, COX-2, MMP-1 and MMP-9	Choi et al., 2010
meso-dihydroguaiaretic acid 	*Machilus thunbergii* Sieb and Zucc	Strongly inhibits MMP-1	Moon and Jung, 2006
Magnolol 	*Magnolia ovovata* Thunb.	Inhibits the NF-kβ-mediated gene expression	Tanaka et al., 2007
1,2,4,6-tetra-O-galloyl-beta-(D)-glucopyranose and 3,4,5-trihydroxybenzoic acid 	*Melothria heterophylla* (Lour.) Cogn.	Inhibits the activity of MMP-1	Cho et al., 2006
Ginsenosides	Panax ginseng L.	Promotes the expression of type-I procollagen gene and protein, prevent MMP-9 gene	Cho et al., 2009 Lee et al., 2009

		induction and elongates the fibrillin-1 fiber. Increase the expression of procollagen type I and decrease MMP-1	
Allylpyrocatechol 	*Piper betel* L.	Protect photosensitization-mediated lipid peroxidation (LPO)	Mula et al., 2008
1,2,3,4,6-penta-O-galloy-d-glucose 	*Terminalia chebula* Retz	Inhibits elastase, hyaluronidase, MMP-2 enzyme and tyrosinase	Kim et al., 2010 Manosroi et al., 2010

2.7. Mutagenicity

Mutagenicity is a process where the structure or amount of genetic material of an organism or cell is changed in a stable and heritable manner. This can happen in nature through using chemicals or radiation (MortelmanS and Zeiger, 2000; Abdelmigid, 2013). Mutagens are chemicals that are responsible for causing mutations; these can include single gene, block of genes or chromosomes. Aneugenicity is the effect of agents that result in a change (gain or loss) in chromosome number in cells; and clastogenicity is the effect of agents that cause structural abnormalities of chromosomes such as chromosome rearrangement or loss (Abdelmigid, 2013).

Chemicals that have the ability to induce mutations even at low exposure levels can result in the damage of germ lines which will affect human health negatively. There are reports stating that somatic mutations which occur in tumor suppressor genes, proto-oncogenes and DNA response genes result in cancer and various other diseases (Abdelmigid, 2013). Damage to DNA which happens in somatic cells has been reported to contribute to accelerated aging, cardiovascular, neurodegenerative disease and immune dysfunction. Germ cell mutations cause infertility, spontaneous abortions and heritable damages to offsprings which will be passed on to the next generation (Mortelmans and Zeiger, 2000). Due to adverse effects that genetic damage has to human health, evaluation of substances that change or modify genetic potential of human beings has become an essential procedure in safety assessments.

Gene mutations are readily measured in bacteria and other cell systems when they result in a change in the growth requirements of the cell, whereas in mammals, chromosome damage in cells is measured by observing the chromosomes under magnification for rearrangements or breaks. The *Salmonella typhimurium*/microsome assay (Salmonella test; Ames test) is a commonly used short-term bacterial assay which is accepted. It identifies substances that have the ability to produce genetic damage that leads to gene mutations. The assay uses a number of *Salmonella* strains with pre-existing mutations that result in the bacteria not being able to synthesize the needed amino acid, histidine. Therefore, not being able to grow and form colonies in its absence. New mutations at the site of the preexisting mutations or genes that are nearby can restore the gene's function and allow the cells to synthesize histidine. These newly mutated cells

can grow in the absence of hidtidine and result in colonies. This is the reason why the test is usually referred to as a "reversion assay" (Mortelmans and Zeiger, 2000).

The *Salmonella* mutagenicity test was explicitly designed to detect chemically induced mutagenesis (Ames, 1975). Throughout the years, this method has been recognized by government agencies, scientific community and corporations. It is used world-wide as an initial screen to detect the mutagenic potential of new drugs or chemicals due to high predictive value for rodent carcinogenicity when a mutagenic response is experienced (Mortelmans and Zeiger, 2000).

2.8. Isolation and characterisation of active compound

The World Health Organization (WHO) states that nearly 20,000 medicinal plants exist in 91 countries including 12mega biodiversity countries. The best steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation. Figure 2.8.1. shows a summary of the overall approaches in extraction, isolation and characterization of bioactive compound from plants extract (Sasidharan et al., 2011).

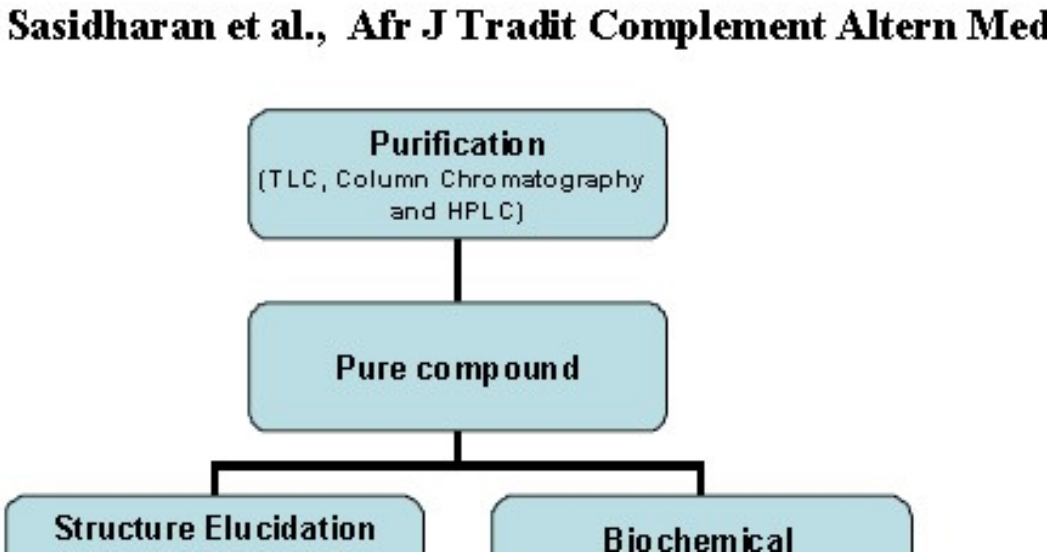


Figure 2.8.1. Summary of the approaches in extraction, isolation and characterization of bioactive compounds from plant extracts (Sasidharan et al., 2011).

2.8.1. Identification and characterisation

Plant extracts usually occur as a combination of several types of bioactive compounds or phytochemicals with different polarities, their separation remains a big challenge for the process of identification and characterization of bioactive compounds. To isolate these different bioactive compounds, different separation techniques can be used such as TLC, column chromatography, flash chromatography, HPLC, size exclusion (sephadex) chromatography. These are used to obtain pure compounds. These pure compounds will then be used to determine the structure and biological activity (Sasidharan et al., 2011).

2.8.2. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a simple, fast, and cheap method that gives the researcher a quick answer as to how many components are present in a mixture. This method is also used to support the identity of a compound in a mixture when the retention factor (Rf) of a compound is compared with the Rf of a known compound. Further tests include the spraying of phytochemical screening reagents, which cause colour changes according to the phytochemicals existing in plants extract; or by viewing the plate under the UV light. This has also been used for validation of purity and identity of isolated compounds (Sasidharan et al., 2011).

2.8.3. Column chromatography

Column chromatography techniques can be utilized for the isolation and purification of bioactive compounds, these can be solid or liquid. This is a solid – liquid method in which the stationary phase is a solid and the mobile phase is a liquid. The rule of column chromatography depends on differential adsorption of a substance by the adsorbent (Virtual Amrita Laboratories Universalising Education, 2018).

The usual adsorbents used in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch and others. Selection of the solvent is based on the nature of both the solvent and the adsorbent. The rate at which the components of a mixture (plant extract) are isolated relies upon the movement of the adsorbent and polarity of the solvent. If the action of the adsorbent is high and polarity of the solvent is low, then the separation is very slow

yet gives a decent separation. If the action of the adsorbent is low and polarity of the solvent is high, the separation is quick yet gives a poor separation, this means that the compounds isolated are not 100% pure (Virtual Amrita Laboratories Universalising Education, 2018).

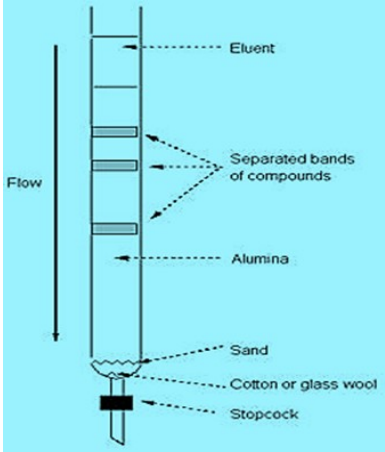


Figure 2.8.2. Separation of compounds using column chromatography (Virtual Amrita Laboratories Universalising Education, 2018).

The adsorbent is made into slurry with an appropriate liquid and put in a cylindrical shaped tube that is stopped at the base by a bit of glass wool or permeable plate Figure 2.8.2. The plant extract to be isolated is dissolved in a suitable solvent and added at the top of the column and is permitted to go through the column. As the extract moves down through the column, the components are adsorbed at various regions depending on their capacity for adsorption. The component with more noteworthy adsorption power will be adsorbed at the top and the others will be adsorbed at the bottom. The different components can be desorbed and collected separately by adding more solvent at the top and this procedure is known as elution. The process of dissolving out of the components from the adsorbent is called elution and the solvent is called is called eluent. The weakly adsorbed component will be eluted more quickly than the other. The different fractions are collected separately. Distillation or evaporation of the solvent from the different fractions yields pure components (Virtual Amrita Laboratories Universalising Education, 2018).

The selection of the solvent system depends on the specific nature of the bioactive compounds that are targeted. The isolation of hydrophilic compounds uses polar solvents such as methanol, ethanol, or ethyl-acetate. For isolation of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some cases, isolation with

hexane is used to remove chlorophyll (Cosa et al., 2006). The compounds of interest can also be non-polar to polar; this is why the method of isolation should be considered (Sasidharan et al., 2011).

2.9. Molecular docking

The molecular docking field has developed in the last three decades, motivated by the need of structure-based drug discovery as well as structural molecular biology. This field has been influenced by the growth in accessibility and power of computers as well as growing access to protein databases and small molecules. The objective of automated molecular software is to comprehend and predict molecular recognition structurally, likely binding modes, binding affinity and energy. In most cases, molecular docking is performed between a target macromolecule and a small molecule, this is known as ligand-protein docking (Morris and Wilby, 2008).

"Re-docking" is the method of taking a known crystal structure of a complex target of interest, separating the ligand and then place back (dock) the ligand into the apo-form of the target. Root mean square deviation (RMSD) is used to measure success, it is the Cartesian coordinates of the atoms of the ligand in the docked and crystallographic conformations. Docking is regarded successful if the RMSD is less than the threshold of 1.5Å. Figure 2.9.1 shows a flow diagram of the steps involved in docking protocols. Docking requires finding the most favourable binding mode(s) of a ligand to the target of interest (Morris and Wilby, 2008).

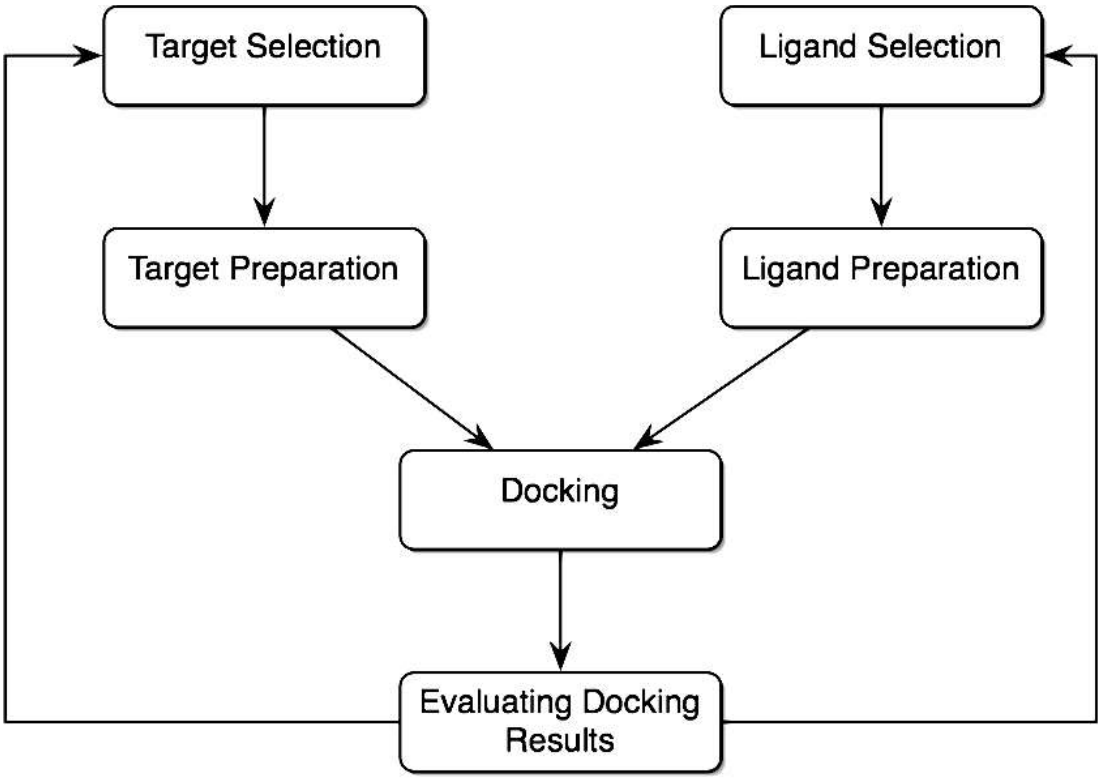


Figure 2.9.1. Flow diagram of the steps involved in docking protocols (Morris and Wilby, 2008).

2.10. Stability of the formulation and extract

2.10.1. Cosmetics

Cosmetic products are used to protect the skin against endogenous and exogenous damaging agents and improve the attractiveness and beauty of the skin (Saraf and Kaur, 2010). The use of cosmetics results in longevity of good, healthy skin and reduces skin disorders besides developing an attractive external appearance (Datta and Paramesh, 2010). In skin care formulations, there are natural or synthetic ingredients present that function in taking care of the health, texture, moisture, elasticity and integrity of the skin by reducing certain components such as free radicals. Therefore, to reduce skin disorders such as hyper pigmentation, skin aging (skin wrinkling) and rough skin texture, cosmetic products are the best choice. There is a huge demand for herbal cosmetics due to the availability of new ingredients, better understanding of skin

physiology, consumer demand and financial rewards for developing successful products (Rajvanshi et al., 2011, Singh et al., 2011). Herbal products are known to have fewer adverse effects compared to products containing synthetic drugs (Ashawat et al., 2009).

2.10.2. Emulsion

An emulsion is a complex mixture of two immiscible phases, with one phase dispersed in another. The macroscopic separation of the phases is prevented by adding a suitable surfactant (Imhof and Pine, 1997). Therapeutic properties and spreading ability of the constituents are increased in an emulsion (Herbert et al., 1988). There are two types of emulsions, oil in water or O/W emulsion is made up of oil droplets dispersed in an aqueous phase. Water in oil or W/O emulsion consists of water droplets dispersed in an oil phase (Timothy et al., 2008). Oil in water emulsions are usually used as water-washable drug bases for general cosmetic purposes, water in oil emulsions are mostly used as emollients and for treating dry skin (Magdy, 2004). Improving the value of these formulations can be achieved by including active ingredients with specific cosmetic effects (Bleckmann et al., 2006). Plants have beneficial and therapeutic properties, their extracts or derived products have often been incorporated in the form of emulsions in recent pharmaceutical and cosmetics preparations and formulations (Khan et al., 2010).

2.10.3. Stability of cosmetic emulsion

Stability tests are an essential part of the testing program for pharmaceutical or cosmetic products due to the fact that instability of the preparation modifies certain parameters such as quality, efficacy and safety (Bilia et al., 2001). Emulsions are thermodynamically unstable and have a tendency to undergo spontaneous change after preparation. Cosmetic emulsions need to have a shelf life of two years. They should be able to with stand extreme weather during transportation from the place of manufacture, to warehouse, to store, and consumer. Therefore, it is important to investigate and predict the stability of an emulsion during the expected shelf life prior to marketing. However, premarket storage test for the entire length of the shelf life of the product (about 2-3 years) are impractical and cosmetic emulsions are subjected to accelerated stability testing in order to predict their shelf life (Bhargava, 2008). Accelerated studies are a good tool as they induce rapid chemical and physical alterations in the formulations (Daudt et al.,

2015). For accelerated stability testing, emulsions are stored at 4°C, room temperature (25°C), 34°C or 40°C, and 45°C or 50°C for three months. This depends on the product category and varies from manufacturer to manufacturer. Emulsions can be subjected to several freeze thaw cycles to determine the effect of extreme cold (Bhargava, 2008). Stability can also be affected by environmental factors such as air, pH and light which can result in the damage of the constituents in the product (Baby et al., 2007). Variation in temperature can change viscosity, solubility, coalescence and hydration. These changes can be detected through the quantification of some components over time by measurement of rheological properties before and after thermal stress. Rheological behavior indicates system quality, usefulness, physical and structural stability (Spiclin et al., 2003; Lippacher et al., 2004; Guaratini et al., 2006). Stability of formulations can also be investigated through the physiochemical and a structural parameters such as pH, phase separation, active ingredient activity and flocculation (Lapasin and Pricl, 1995; Korhonen et al., 2001; Tadros et al., 2004; Casteli et al., 2008; Borella et al., 2010; Gianeti et al., 2012).

Sensory analysis is another essential approach, this is a multidisciplinary science that includes measurement, interpretation and understanding of the human responses to product properties detected through the senses. Sensory evaluation data has been used as part of the marketing decision in the cosmetic industry, it has also been demonstrated to be of considerable importance in the development of cosmetic products designed to delight the consumer's senses. Furthermore, sensory evaluation data has been used to determine the acceptance of a particular product and to optimize an existing product (Martens, 1999; Jog et al., 2012;).

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Chapter 3
Plant selection

CHAPTER 3

Elegia tectorum L.F. Moline and H.P. Linder.

Scientific name*:* *Elegia tectorum* L.F. Moline and H.P. Linder.

Family: Restionaceae

Common names: Cape Thatching Reed (English), Danriet (Afrikaans)

Synonym: *Chondropetalum tectorum* (L.f.) Raf.

Morphology: Amphiphyte - Plant that grows on the edges of water or wetlands, and is sometimes submerged

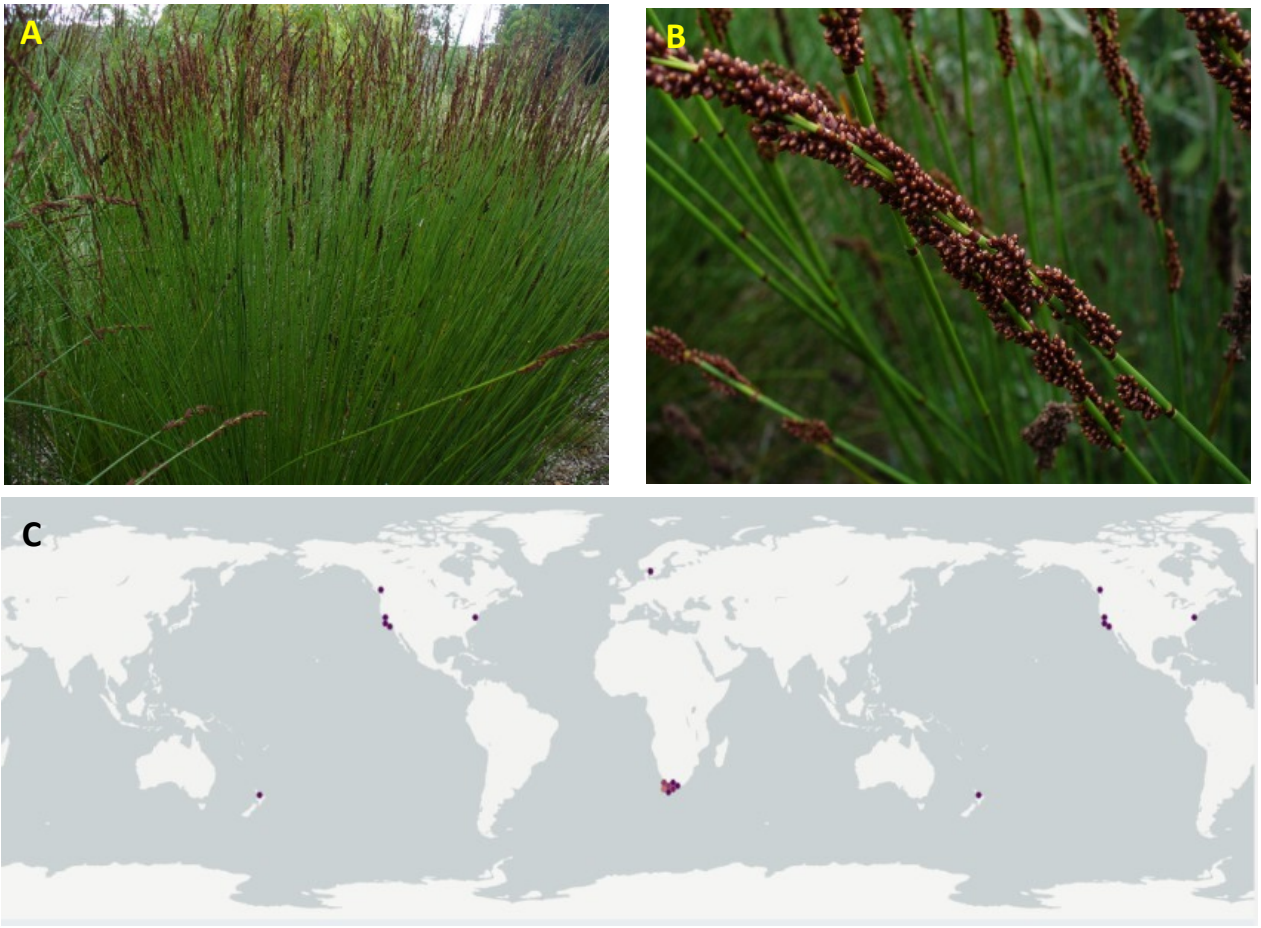

Figure 3.1.1. A) *Elegia tectorum* aerial plant part (Massyn, 2007), B) flower (Massyn, 2007), C) Global distribution (GBIF, 2017).

3.1. Botanical characteristics

Elegia tectorum is a perennial species, belonging to the Restionaceae, that has an upright, tufted reed- like appearance. It is symmetrical, with dark green, thin stems which have dark brown flowers at the top, which occur in autumn (Figure 3.1.1. A and B). This plant can grow to approximately one to one and half meter tall and spread up to two to three meters (South African National Biodiversity Institute, 2016).

3.2. Geographical distribution

Elegia tectorum is found mainly in the Western Cape fynbos region. Naturally it occurs in marshes (wetland that is dominated by herbaceous rather than woody species) and seeps on deep sand. It can occur in the Western and Eastern Cape extending from Clan-William to Port Elizabeth (South African National Biodiversity Institute, 2016). It is part of the vegetation in areas that are rich in fauna and flora (SA-Venues, 2019) (Figure 3.1 C).

3.3. Ethnobotanical usage

No medicinal uses have been reported form this species, however it is commonly used as a thatching reed. Other members of this family are traditionally used for treating itching skin and boils (Wiart, 2006).

3.4. Genome sequencing

The following genes have been completely or partially sequenced and can be found on the NCBI website under nucleotide sequences for *E. tetorum* (NCBI, 2019)

- BSTR_2021137 putative LOV domain-containing protein mRNA

- BSTR_2018403 putative LOV domain-containing protein mRNA
- Ribulose-1,5-bisphosphate carboxylase/oxygenase, subunit (rbcL) gene
- CtPIN1-alp PIN-like protein mRNA
- NSW:276675 trnK gene and maturase K (matK) gene
- BSTR_2021136 putative LOV domain-containing protein mRNA
- BSTR_2009893 putative LOV domain-containing protein mRNA
- phytochrome B (PHYB) gene
- atpB-rbcL intergenic spacer
- tRNA-Leu (trnL) gene, trnL-trnF intergenic spacer, tRNA-Phe (trnF) gene
- 1/MADS5-like protein (L1O5) mRNA

3.5. Chemical constituents

Compounds present within *E. tectorum* have not yet been identified. Compounds present within species belonging to the same family have been listed (Table 3.5.1).

Table 3.5.1. Flavonoids in South African Restionaceae (Harborne, 1979; Harborne 2000; Harborne and Williams, 2001)

Flavonol glycosides	Flavones and other constituents
Quercetin-3-glucoside	Luteolin
Quercetin -3-galactoside	Apigenin
Quercetin -3-rutinoside	Chysoeriol
Myricetin-3-galactoside	Tricin
Larycitrin-3-galactoside	Hypolaetin
Syringetin-3-galactoside	Flavonols
Myricetin-3-arabinoside,	Myricetin
Myricetin-3- rhamnoside	Quercetin
Larycitrin-3-galactoside.	Kaempferol
Larycitrin-3-diglycoside	Gossypetin

3-diglycoside	Gossypetin 7-methyl ether
Gossypetin 7-methylether	Larycitrin
Flavonol glycoside	Syringetin
Sulfatoglucoside	Herbacetin 4'-methyl ether
7-Sulfatogalactoside	Quercetin 3-methyl ether
7-Sulfatoglucuronide	Iso-orientin
7-Sulfate-8-glucoside	Chrysoeriol-3-glycoside
Hypolaetin 7-methyl ether	Apigenin and Chrysoeriol-glycosides
3 -Sulfatogalactoside	Luteolin-glycoside
3 -Sulfatoglucuronide	Flavone glyc
	Glycoflavones
	Luteolin-7-glucoside,
	Luteolin-diglycoside
	Chrysoeriol-7-diglycoside
	Orientin
	iso-orientin
	lucenin
	Luteolin-5-glucoside
	Procyanidin

3.6. Biological activity

No biological activity has been reported on *E. tectorum.* One plant in the genus *Elegia* has biological activity reported. Seventy one extracts prepared from *Elegia nuda* were investigated for their hemolytic activity, however only three extracts showed significant activity, the branch methanol and water extracts showed hemolytic activity at a concentration of 250 µg/ml and leaf methanol extracts showed hemolytic activity at a concentration of 500 µg/ml. The positive control was saponin which had a total hemolytic activity at a concentration of 20 µg/ml (Oliveiral et al., 2009). No biological activity has been reported on other plants in the same genus or same family.

3.7. Plant collection

Elegia tectorum L.F. Moline and H.P. Linder aerial plant part was collected at the University of Pretoria, Hatfield Campus, Pretoria in February 2018. It was identified and authenticated by Ms Magda Nel at the H.G.J.W. Schweickerd Herbarium (University of Pretoria, South Africa) and the herbarium specimen (PRU 122257) was deposited.

3.8. Extract preparation

Plant material was collected and rinsed with distilled water to remove unwanted substances. The aerial part of the plant was frozen at -80°C for three days and then freeze dried to eliminate all the water from the plant material. The dried plant material was ground to a fine powder using an IKA grinder (MF 10.1 Head 2870900) using 2mm head and then weighed. Sequential extraction took place in the following manner: 200g of the powder was added to 900 ml hexane and left on a shaker (Labcon Shaker 308611) for a three days. The plant material was filtered through a Büchner funnel using a Whatman No. 1 filter paper. The hexane filtrate was evaporated using a rotary evaporator (Büchi Rotavapor B-480) which resulted in concentrated extract. This was repeated 12 times until the solvent was no longer green when exposed to the powdered plant material. The same powered plant material that was used to extract with hexane was used to extract with dichloromethane (900 ml, 8 times), ethyl acetate (900 ml, 4 times) and lastly methanol (900 ml, 4 times). Non sequential extraction was conducted for acetone, water and ethanol (new plant material was used with each solvent respectively). The procedure was similar to the one mentioned above for hexane. The extracts were then placed in a fridge at 4°C to be used for subsequent experiments.

3.9. References

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Chapter 4
Biological activity

CHAPTER 4

Reversing the effect of skin aging using *Elegia tectorum* (L.F) Moline & H.P. Linder *

4.1. Abstract

Skin aging is associated with the degradation of the extracellular matrix through increased activity of enzymes such as elastase, collagenase and hyaluronidase. The aim of this study was to investigate the ability of a South African wetland plant, *Elegia tectorum* to reduce the formation of wrinkles on the skin through the inhibition of elastase and KIAA1199 protein. Elastase inhibition assay was used to screen the plant extracts of *E.tectorum* made from ethanol, hexane, dichloromethane, ethyl acetate, water, acetone and methanol. The methanolic and ethanolic extracts showed highest anti-elastase activity with an inhibitory concentration (IC_{50}) of 10.93±4.98 and 13.495±1.53 µg/ml respectively. The ethanolic extract, which is a suitable solvent in product development, was selected for further testing. *In vitro* cytotoxicity was investigated on human colorectal adenocarcinoma cell line (HT-29), the ethanolic extract was found to be not toxic at the highest tested concentration (IC_{50}>400 µg/ml). Furthermore, at non-toxic concentrations (15, 60, and 240 µg/ml), *E.tectorum* was able to significantly inhibit the KIAA1199 protein. The mutagenic potential of the extract was investigated using *Salmonella typhimurium* TA98, and was found to be a non-mutagen. Molecular docking was conducted to predict the binding affinity and binding mode of the compounds, identified through GC-MS, to the active site of elastase. Five compounds had the closest docking score to the reference ligand which had a score of -11.64, octadecanoic acid, 9,12,15-octadecatrienoic acid (Z,Z,Z), n-hexanoic acid, 3-(5-methylfuryl)-n-furamidopropionamide, and hexanedioic acid bis(2-ethylhexyl) ester. The docking scores were -6.92, -6.39, -6.20, -5.21, -5.02 respectively. Bio-assay guided fractionation column chromatography was conducted and none of the six pooled

fractions were able inhibit elastase, indicating a potential-synergistic activity with two or more compounds within the crude extract. Stability testing of the formulation containing the extract and extract was conducted and parameters such as odour, colour, pH, and viscosity were investigated. The results indicated that the product is stable for a period of two years when stored at temperatures below 40 ºC and away from direct sunlight. *In vivo* irritancy studies revealed that the plant extract, when applied neat, showed mild irritancy. *Elegia tectorum* was able to inhibit elastase enzyme and KIAA1199 protein. It is a good candidate as an anti-wrinkle product as it is a non-mutagen, is stable and is a mild irritant. Therefore, this plant has a potential to result in an antiaging product.

* Chapter 4 has been written in a manuscript format

4.2. Introduction

All living organisms undergo a process known as aging. During this process the skin is the most visible tissue that is affected. Skin aging is associated with the degradation of the extracellular matrix (ECM) through increased activity of enzymes such as elastase, collagenase and hyaluronidase. Therefore, this implies that the level of elastin, collagen and hyaluronic acid decreases resulting in a loss of flexibility and strength of skin, and hence wrinkle formation. The focus of this project was to investigate the inhibition of hyaluronic acid depolymerization via the KIAA1199 protein instead of the commonly employed hyaluronidase enzymes using wetland plants. The reason for this was that hyaluronidase enzymes have important functions in the skin, for example the depolymerization of hyaluronic acid into a high molecular weight hyaluronic acid plays a role in anti-inflammation and reduces the formation of scars. If hyaluronic acid is degraded into a low molecular weight molecule, it improves the synthesis of type I and VIII collagen which are structural ECM molecules. The KIAA1199 protein depolymerizes hyaluronic acid independent of the hyaluronidases or the CD44 receptor. This study also looked at the elasticity of the skin by focusing on the anti-elastase potential of the wetland plants. Mutagenicity, molecular docking column chromatography, stability of the extract and formulation, and irritancy patch studies were conducted.

4.3. Materials and methods

Materials

Porcine pancreatic elastase, Trizma base, N-Succinyl-Ala-Ala-Ala-p-nitroanilide, Ursolic acid, methanol were purchased from Sigma-Aldrich. HT29 cell line, trypan blue, McCoys media, fetal bovine serum (FBS), Penicillin-Streptomycin-Fungizone antibiotics (PSF), T57 culture flasks, phosphate buffer saline (PBS), trypsin-EDTA (0.25%), haemocytometer. Coated 96-well Strip Plate, Standard (Lyophilized), Sample Diluent, Assay Diluent A, Assay Diluent B, Detection Reagent A, Detection Reagent B, Wash Buffer (30x), TMB Substrate, Stop Solution, Adhesive Plate Sealers, Instruction Manual, Microplate reader with 450nm wavelength filter, High-precision pipette and sterile pipette tips, Eppendorf tubes, 37°C incubator, Deionized or distilled water, Absorbent paper, HT29 cells, centrifuge, Phosphate-buffered saline (PBS), ultra-sonicator, plant extract, 24 well plate were purchased from LifeSpan BioSciences, Inc. Demineralized water, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA NA_2), carbopol 934, triethanolamine (TEA 99%), BHT, arlacel 165, kotilen s1, ceto stearyl alcohol, kosteran s1, mineral oil, silicone 200/100, lanolin, moist 24, witch hazel distillate, kemaben, glycerine, and vitamin E acetate were purchased from Botanichem CC. Cotton buds, demineralised water, 8mm finn chambers, finn chamber templates, black marker pens, positive displacement pipette, sodium lauryl sulphate solution (1%) as a positive control, demineralised water as a negative control, transpore tape and 1CC Syringes.

Plant collection

The aerial part of *Elegia tectorum* L.F. Moline and H.P. Linder was collected in February 2018 at the University of Pretoria, Hatfield Campus, Pretoria. The herbarium specimen (PRU 122257) was deposited and the plant was identified and authenticated by Ms Magda Nel at the H.G.J.W. Schweickerd Herbarium (University of Pretoria, South Africa).

Plant extraction

To remove unwanted substances on the plant material, it was rinsed after collection, frozen at -80°C for three days then freeze dried to remove all the water from the plant material. The dried plant material was ground to a fine powder Using an IKA grinder (MF 10.1 Head 2870900)

using 2mm head and then weighed. Sequential extraction was followed in the following manner: 200g of the powder was added to 900 ml hexane and left on a shaker (Labcon Shaker 308611) for three days. The plant material was filtered through a Büchner funnel using a Whatman No. 1 filter paper. The hexane filtrate was evaporated using a rotary evaporator (Büchi Rotavapor B-480) which resulted in concentrated extract. This was repeated 12 times until the solvent was no longer green when exposed to the powdered plant material. The same powered plant material that was used to extract with hexane was used to extract with dichloromethane (900 ml, 8 times), ethyl acetate (900 ml, 4 times) and lastly methanol (900 ml, 4 times). Non sequential extraction was conducted for acetone, water and ethanol (new plant material was used with each solvent respectively). The procedure was similar to the one mentioned above for hexane. The extracts were then stored in a fridge at 4°C to be used for further experiments.

Elastase inhibition

The elastase inhibition potential of the different samples was evaluated using the elastase inhibition assay, as described by Bieth et al., 1978 with modifications. Tris buffer was prepared and had a concentration of 0.012 g/ml (pH 8). The porcine pancreatic elastase enzyme was prepared to stock 1 solution of 12 U/mg, stock 2 was prepared and tested at a concentration of 0.0416 U/mg. N-succinyl-(Ala)3-p-nitroanilide (the substrate) was prepared to a stock concentration of 1.81 mM and tested at a concentration of 0.32 mM. The positive control ursolic acid was prepared to a stock concentration of 2000 µg/ml in methanol and serially diluted to yield a test range of 250-7.81 µg.ml. Similarly, the test samples were prepared to stock concentrations of 2000 µg/ml in methanol and serially diluted to a test concentration range of 250-7.81 µg/ml in triplicate. The experiment was repeated three times and the plates were read kinetically for 15 minutes at 405 nm at a temperature of 37ºC using KC Junior software and the results were analyzed using GraphPad Prism 4 in order to obtain the IC_{50} values.

Cell culture

Human colorectal adenocarcinoma cell line (HT-29) was cultured in McCoys media supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100U/ml penicillin, 100 μg/ml streptomycin and 250 µg/ml fungizone). The cells were kept in a humidified incubator set at 5% CO_2 at 37ºC to grow. Cells were subcultured for 10 minutes by treating them with trypsin-

EDTA (0.25% trypsin containing 0.01% EDTA). Using a hemocytometer, the cell number and viability were evaluated by exclusion of trypan blue dye. Cells were seeded in 24-well plate at a density of 1×10^5 cells/well, then the cells were looked under a microscope for morphological assessment.

In vitro cytotoxicity

Cytotoxicity was measured using the Presto-Blue assay as described by (Lall et al., 2013). The cells were seeded in a 24-well microtiter plate at a concentration of 1×10^5 cells/ml, the cells were incubated and allowed to attach for 24 hours. The cells were exposed to the drug actinomycin D which was a positive control with concentration between 0.002 µg/ml to 0.5 µg/ml. The cells were also exposed to the *E.tectorum* extract at a concentration ranging between 1.563 µg/ml to 400 µg/ml. The microtiter plate was incubated for 72 hours and 20 µl of Presto-Blue was added. The plates were incubated for another 2 hours where after the absorbance of the colour complex was read at 570 nm with a reference wavelength at 600 nm, using a BIO-TEK Wave XS multi-well reader. This assay was carried out in triplicate to calculate the IC_{50} of the cell population. GraphPad Prism 4 software was to analyze the results.

KIAA1199 protein inhibition

In two 24 well plate, 1×10^5 cells/well was plated and incubated to grow for 24 hours at 5% CO_2 at 37ºC to allow for adherence. Three different non-toxic concentrations of the plant extracts were tested from the lowest to highest, 15, 60 and 240 µl/ml, the lowest concertation was selected based on the IC_{50} of the ethanolic extract obtained in elastase inhibition assay, and it was increased by a factor of 4, cells in media only were used as a control. The plates were incubated for a further 24 hours at 5% CO_2 at 37ºC. To ensure that the cells were still viable, in one plate cell viability was determined by adding 100 µl of Presto-Blue to all the wells, the plate was incubated for further two hours and viewed under a microscope for morphological assessment. In the remaining plate, the cells were centrifuged in order to ensure that the contents of the cells remain as the pellet at the bottom of the plate and the supernatant which was mostly the media was transferred into a 96 well plate using a pipette and discarded. The pellet was washed three times with PBS, the cells were still attached at the bottom of the 24 well plate, the cells were further resuspended in PBS to detach them from the plate. After detaching, they were

then lysed by ultra sonication for 10 minutes and were further centrifuged at 1500xg for 10 minutes at 2-8ºC, the supernatant and pellet were collected, these were put in the -80ºC freezer for 24 hours.

Determining the KIAA1199 content was done according to LifeSpan BioSciences, Inc, catalog no LS-F7390. The standard stock solution of KIA1199 protein (recombinant protein) was 20 ng/ml, one tube of the standard was resuspended in 1ml of sample diluent. This was incubated at room temperature for 10 minutes, with gentle agitation to avoid foam. In eight Eppendorf tubes, 250 µl of the sample diluent was added. The standard stock was then serially diluted, pipetting 250 µl in each Eppendorf tube. The concentration in each of the Eppendorf tube was 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.3313 ng/ml, 0.157 ng/ml and the last tube was the zero standard (0 ng/ml) where it was only the sample diluent. The stock detection reagents A and B were brought to room temperature (18-25ºC). These were diluted to a ratio of 1:100 using the assay diluent A and B. Therefore, 52 µl of detection reagent into 5148 µl of assay diluent. 600 ml of the 1x wash buffer was also prepared in the following manner: 20 ml of the wash buffer concentrate was diluted with 580 ml of distilled water. The substrate was prepared according to the number of wells in the plate that were going to be used, this was light sensitive. All reagents were mixed well before pipetting. The standard (100 µl), blank and sample (supernatant, pellet), per well were added then covered with a plate sealer and incubated for 1hour at 37ºC. The sample included the plant extract tested at a concentration of 15, 60 and 240 µl/ml, media and cells as a control. The liquid was then removed using a pipette, making sure that the bottom of the plate is not touched. Detection reagent A working solution (100µl) was added to each well, the plate was covered with a plate sealer and gently agitated to allow thorough mixing, and this was incubated for 1hour at 37ºC. The liquid was aspirated from each well and washed three times by adding approximately 350 µl of 1x wash buffer using a multi-channel pipette. Each wash was allowed to sit for 1-2 minutes before the next aspiration. After the last wash, the wash buffer was completely removed, and the plate was inverted and tapped against the absorbent paper. Detection reagent B working solution (100 µl) was then added to each well, again covered with a plate sealer and incubated for 1hour at 37ºC. The liquid was aspirated then followed by washing five times as previously outlined. The TMB substrate solution (90 µl) was added to each well. The plate was covered with a new plate sealer and incubated for 10-20 minutes at 37ºC.

The plate was covered with a foil to protect from light and it was monitored periodically until optimal colour development occurred. A stop solution (50 µl) was added to each well. This resulted in colour change from blue to yellow immediately. The stop solution was added in the wells in the same order and timing as the TMB substrate solution. The optical density (OD value) of each well was determined using PerkinElmer multimode plate reader VICTOR Nivo set to 450 nm. GraphPad Prism 4 software was to analyze the results.

Mutagenicity

Preparation of the extract for mutagenicity testing

The extract was prepared to a stock concertation of 5000 µg/ml in 10% DMSO, it was further diluted with sterile 10% DMSO resulting in two lower concentrations of 5000 and of 500 and 50 µg/ml.

In vitro mutagenicity of the extract using the Ames test

Salmonella microsome assay was used to investigate the mutagenicity of the extract in a histidine deficient growth medium. Using the Ames test, the extract was incorporated with *Salmonellla typhimurium* tester strains TA98 with no metabolic activation. This method was conducted based Maron and Ames (1983), then revised by Mortelmans and Zeiger (2000). The bacterial stock (100 µl) was incubated in 20 ml of the Oxid No.2 nutrient broth at 37 ºC on a shaker for 16 hours. The cultured bacteria (100 µl), was added to 100 µl of the plant extract with 500 µl of phosphate buffer and 2ml of the top agar containing biotin-histidine (0.5mM). This mixture was vortexed then transferred on the surface of minimal agar plate then further incubated for 48 hours at 37 ºC. Sterile distilled water was used as a negative control and 4-nitroquinoline-1-oxide (4-NQO) was used as a positive control at a concentration of 2 µg/ml. The sample was tested in triplicate. After incubating for 48 hours, the number of bacterial colonies was counted manually and the results were presented as the mean (±standard error) number of the revertant colonies per plate. By looking at the background bacterial growth, mutagenicity of the extract was identified; if growth was absent then then no toxicity and if growth was present then there is toxicity.

Molecular docking

Structure selection and protein preparation

The crystal structure of the elastase enzyme was selected for computational analysis (PDB ID: 1U4G). The enzyme was co-crystalised with N-(1-Carboxy-3-phenylpropyl) phenylalanyl-alpha-asparagine (HPI) in the active site. Schrödinger's Protein Preparation wizard was used for pre-processing of the complex in order to assign bond orders, completion of missing side chains, addition of hydrogens and as well as loops. PROPKA was used to optimise hydrogen bonds at a pH of 7.0 followed by the minimisation of the complex using OPLS force field (Harder et al., 2016). The ethanol extract was sent to the University of Pretoria Chemistry department for gas chromatography–mass spectrometry (GC-MS) analysis to identify the probable compounds that are present in the extract. The simplified molecular input line entry system (SMILES) of the compounds were obtained.

SiteMap

SiteMap was used to identify and confirm possible target sites within the crystal structure of elastase (Schrödinger, LLC, NewYork, NY, 2018; Halgren, 2007, 2009). Binding sites on Sitemap were predicted using algorithms based on functionality, solvent exposure and size.

Molecular Docking -Glide

Glide was used for docking the ligand (E.tectorum compounds) to the elastase enzyme site in order to estimate the binding affinities (Halgren et al., 2004) .

Isolation

Liquid partitioning:

Crude ethanol extract (100mg) was dissolved in 300ml dH_2O until it completely dissolved. It was then added in the separating funnel; ethyl acetate (250ml x3) was added, mixed and allowed to settle resulting in two layers. The water fraction was at the bottom, the ethyl acetate fraction was at the top. The water fraction was collected then *n*-butanol (250ml x 3) was added to it in a separating fennel. Then the water fraction was collected at the bottom while the *n*-butanol fraction was at the top. There were three fractions in total after this, ethyl acetate, *n*-butanol and water. These were concentrated using a rotary evaporator apparatus (BUCHI Rotavapor B-480). The water and butanol fractions were concentrated by making azeotropic mixture (butanol-water, 60:40, v/v). The fractions were further tested for elastase inhibition.

Slurry preparation using the water fraction:

The water fraction was dissolved in methanol (minimal amount), silica was added and left to dry overnight. This mixture was grinded using mortar and pestle to a fine powder.

Column chromatography:

Cotton wool was placed at the bottom of a column. A slurry of 500g of silica in *n*-hexane was placed into the column and allowed to settle. Then the previously prepared dried slurry of water fraction and silica was added into the column. Fractions were collected (265 in total), the first fraction was eluted with hexane. The other fractions were eluted in the following manner: fraction 2-11 with ethyl acetate, fraction 12- 40 with 2% methanol in ethyl-acetate, fraction 41-62 with 5% methanol in ethyl-acetate, fraction 63-181 with 8% methanol in ethyl-acetate, fraction 182-215 with 10% methanol in ethyl-acetate, fraction 216-237 with 15% methanol in ethyl-acetate, fraction 238-250 with 20% methanol in ethyl-acetate, fraction 251-260 with 30% methanol in ethyl-acetate and fraction 261-265 with 50% methanol in ethyl-acetate. Based on their TLC profile, fractions were pooled together into six sub-fractions (F1-12, F13-21, F22-62, F63-175, F175-250, F251-265). As evident for the TLC profiles (Figure 4.4.7), fraction 22-62 had a major compound. This compound was further purified by precipitation with ethyl acetate. The pooled fraction was dissolved into a minimum amount of methanol and ethyl acetate was added drop by drop. This led to the precipitation of the pure compound. The precipitated compound was again dissolved into the methanol and the same process was repeated for five times. Finally, the precipitated compound was dissolved into methanol and examined for its TLC profile. The TLC showed a single spot which confirmed the purity of the compound. This purified compound was then submitted for the NMR analysis

Stability

Preparation of the extract

300 mg of the ethanol extract was dissolved in 50ml made up 40% ethanol and 60% water, with a final concentration of 6 mg/ml.

Water phase: Demineralised water (0.9055 kg) was heated in a pot until it reached 75ºC. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA NA_2) (0.0015 kg), glycerine

(0.030 kg), and carbopol 934 (0.0015 kg) were added into the hot pot respectively until dissolution. Triethanolamine-99% (TEA 99%) (0.0015 kg) was added into the mixture in the pot, these were mixed for 20 minutes until the mixture was lump free, thick and clear.

Oil phase: A glass beaker was placed on a hot plate and the temperature was increased to 75ºC then the following was added respectively until dissolved: arlacel 165 (0.030 kg), ceto stearyl alcohol (0.0225 kg), BHT (0.0015 kg), mineral oil (0.090 kg), kotilen S1 (0.015 kg), kosteran S1 (0.034 kg), lanolin (0.030 kg) and silicone 200/100 (0.0225 kg). The oil phase was added to the water phase at 75ºC and a hand blender was used to mix until the mixture cooled to 40ºC. Kemaben (0.015 kg), moist 24 (0.045 kg), vitamin E acetate (0.030 kg) and witch hazel distillate (0.075 kg) were added to the bulk at 40ºC. A hand blender was used to mix until the formulation cooled to room temperature.

The dissolved ethanolic extract (10%) was added to the cooled formulation. The formulation (250 g) was then placed into four different jars, these were incubated at four different temperatures (4ºC, 25ºC, 40ºC and 50ºC). Parameters such as odour, appearance, pH and viscosity were investigated over a period of 12 weeks (week 1, week 2, week 4, week 8 and week 12). The remaining dissolved ethanolic extract (25 ml) was also placed into four different jars and exposed to four different temperatures (4ºC, 25ºC, 40ºC and 50ºC). The extracts were run on TLC plates on week 1, week 2, week 4, week 8 and week 12.

Irritancy patch test

The irritancy patch test was done on the inner forearm of twenty (20) subjects. The ethanolic plant extract and placebo (distilled water) were applied topically using the 1CC syringes and placed on the skin in Finn chamber (25 x 10 mm) and kept in position for 24 hours until evaluation. Visual assessments of the test sub-sites were conducted for any irritant reaction after 24, 48 and 72 hours. Reactions on the skin were graded as follows: 0 = no response, 0.5 = minimal/doubtful response, 1 = mild erythema, spotty or diffuse, 2 = definate erythema, uniform redness, itching or burning response. swelling may occur, 3 = strong and severe uniform redness, swelling or spreading beyond the area of the disc may occur, 4 = fiery redness, oedema, papules, bullae

Metal toxicity

The extract was evaluated for the presence of metal contaminants. It was sent to Merieux NutriSciences Chelab S.R.L. Metal contaminant detection was done for Arsenic, Cadmium, Mercury and Lead.

4.4. Results and discussion

Elastase inhibition

The inhibitory effect of *E. tectorum* seven extracts from different solvents were investigated. (Figure 4.4.1). There is no literature that indicates that this genus or plants in this family have been tested against the elastase enzyme. The positive control ursolic acid is a known inhibitor of the enzyme, it had an IC_{50} of 6.04 µg/ml. From non-polar to polar extracts: hexane had and IC_{50} of 67.47 µg/ml, dichloromethane 66.11 µg/ml, acetone 21.79 µg/ml, ethyl acetate 86.19 µg/ml, ethanol extract 14.58 µg/ml, methanol 10.93 µg/ml, and water 51.14 µg/ml. The methanolic and ethanolic extracts had the best inhibitory activity, as their IC_{50}'s were the lowest. The acetone extract showed good inhibitory activity even though it was not as good as methanol and ethanol. The other extracts did not have good elastase inhibition such as ethyl acetate, hexane and dichloromethane compared to the positive control.

Kim et al., (2009) investigated elastase inhibition of *Callistemon lanceolatus* ethanol extract, it showed good anti-elastase activity with an IC_{50} of 20.2 µg/ml. In another study, Schmidt et al., (2009) studied elastase inhibition of Brazilian plants, one of them was *Iresine herbstii* hexane and ethanol extracts. These had an IC_{50} value of 59.68µg/ml and 13.02 µg/ml respectively.

In 2016, Bravo et al., evaluated elastase inhibition of *Ugni myricoides* extracts and fractions. The ethyl acetate fraction had an IC_{50} of 80.8 µg/ml while the acetone extract was 33.8 µg/ml. Another study by Paya et al., (1996) showed elastase inhibition of *Phyllanthus sellowianus* in different solvents. The dichloromethane fraction had an IC_{50} of 68.6 µg/ml; the methanol fraction was 16.6 µg/ml. Another plant that was investigated was *Gamochaeta simplicicauli*, the dichloromethane fraction had an IC_{50} value of 88.3 µg/ml and methanol was 16.2 µg/ml. *Psidium guajava* water extract showed an IC_{50} of 44 µg/ml (Mendez et al., 2015). All these IC_{50}

values are within the range of the IC_{50} values obtained in this study although the plants are different. The effect may have been due to the solvents used which determine the type of compounds extracted, another influence on the activity is that the plants were different and plant parts which were used were different.

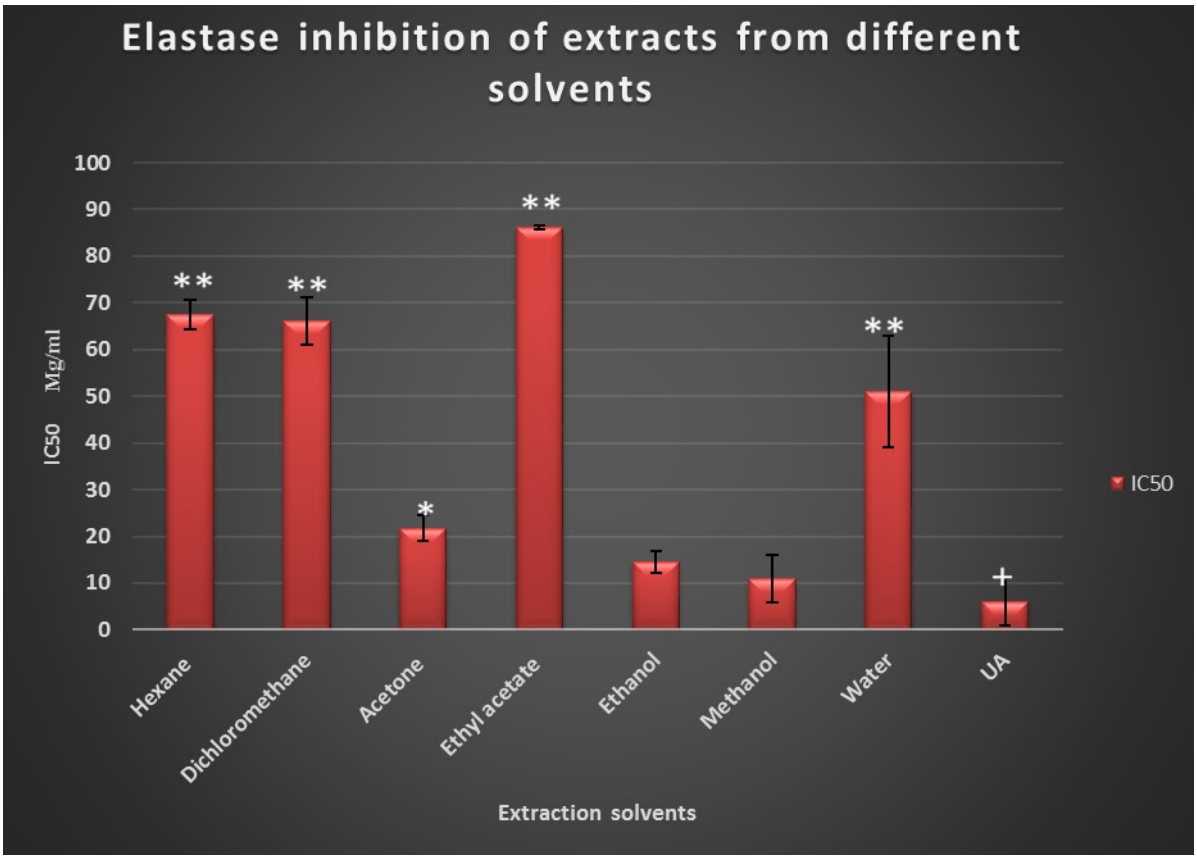


Figure 4.4.1. Elasatse inhibition of extracts made from different solvents. Data given as mean IC_{50} ± SD (n = 3), UA-ursolic acid.

Cytotoxicity

Human colon adenocarcinoma (HT-29) cell line was used in this study because Birkenkamp et al., (2002) reported that the KIAA1199 transcript was strongly up-regulated in these cells. In another study, the KIAA1199 protein expression was also found to be up-regulated in colon adenomas (Sabates et al., 2007). Other researchers have also proved an increase in protein expression of KIAA1199 in colon cancer tissues and cells, as well as down regulation by knockdown or suppressing the gene (Birkenkamp et al., 2011, Zhang et al., 2017). Knockdown

of β-catenin mediated by siRNA in LS174 colon cells decreased KIAA1199 transcript expression (Sabates et al., 2007). In the skin, KIAA1199 protein depolymerizes hyaluronic acid resulting in reduced hydration which is a major factor in aging skin, therefore in the present study, the HT-29 cells were selected based on that the KIAA1199 protein is highly expressed.

The cytotoxicity of *E.tectorum* ethanolic extract was evaluated and the IC_{50} values were obtained. The extract had an IC_{50} of >400µg/ml, this indicated that it was non-toxic to HT-29 (human colon adenocarcinoma) cell line. The results were restricted to a value between 0-400µg/ml, therefore an IC_{50} that is greater than 400µg/ml means that the sample produced an IC_{50} greater than the highest concentration tested and can thus be considered non-toxic. Actinomycin D was used as a positive control and it had an IC_{50} of <0.005µg/ml, it is known to be toxic and any IC_{50} value that is close to actinomycin D would indicate that the sample is toxic. There was no literature found concerning the cytotoxicity of *E. tectorum* on HT-29 cell line. Figure 4.4.2. shows the plate layout.

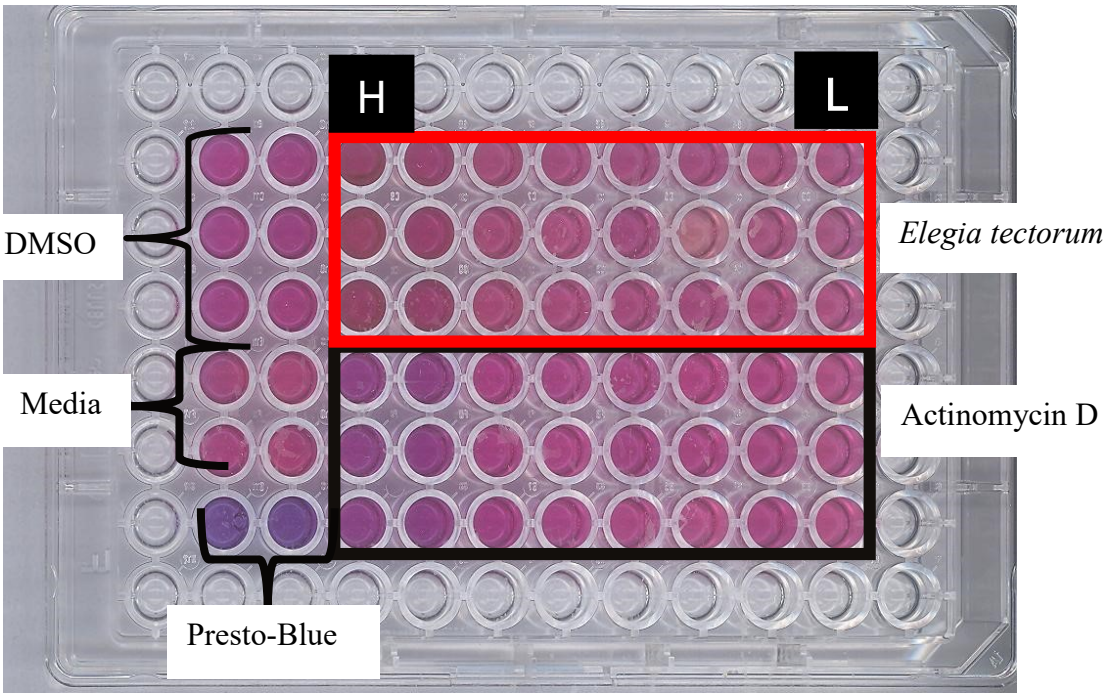


Figure 4.4.2. Growth indicator Presto-Blue on HT-29 cells in the presence of McCoys media. The plate contained *E.tectorum* ethanolic extract, DMSO -dimethyl sulfoxide, actinomycin-D-positive control. H-highest concentration, L-lowest concentration.

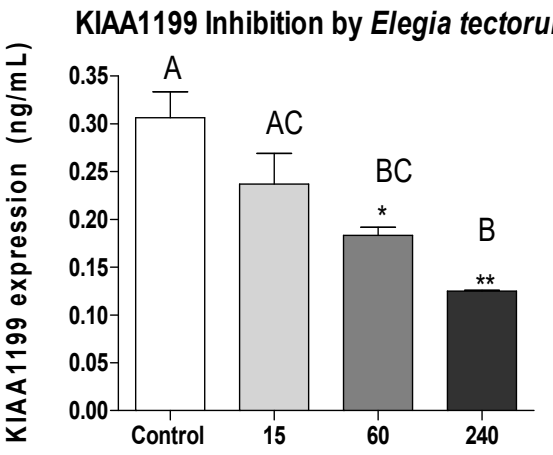
KIAA1199 Protein inhibition

Hyaluronic acid is an important component of the extracellular matrix in the skin. Hyaluronic acid reduction in the dermis and overexpression of the KIAA1199 protein, a major molecule for HA degradation in skin fibroblasts, causes facial skin wrinkling (Yoshida et al., 2019[a]). GraphPad Prism 4 software was to analyze the results, the standard was used as a control. Figure 4.4.3 shows KIAA1199 protein expression in HT-29 cell line after treating with ethanolic extract of *E.tectorum*, that there was no significant difference between the control and the low concentration of *E.tectorum* (15µg/ml). There was significant difference between the control, 60 and 240 µg/ml which indicated that the KIAA1199 protein was inhibited. There was non-significant relationships (between 15 and 60 µg/ml, AND 60 and 240 µg/ml). Therefore, two significant differences ($p < .05$ at 60 µg/ml and $p < .01$ at 240 µg/ml as measured against the control) were observed. The greater significant difference between 240 µg/ml and the control showed that the higher the concentration of the plant extract used, the more the protein was inhibited. There was no literature that evaluated KIAA1199 protein inhibition using plant extracts for wrinkle reduction using the Human CEMIP/KIAA1199 ELISA Kit. Inhibiting KIAA1199 protein especially in fibroblast cells, will reduce the quantity of hyaluronic acid molecules that are depolymerized in the skin, thus reducing wrinkle formation.

Yoshida et al., (2019)[a] , studied the inhibition of *Geranium thunbergii* by culturing fibroblast cells (Detroit 551) for 48 hours with high molecular weight HA in the presence of *G. thunbergii* extract (0, 0.1, 0.3, 1 or 3 mg/ml). The collected culture media was put through gel filtration chromatography (size-exclusion chromatography). The cells degraded HA of >1000 kDa to inter-mediate-sized HA of 10-100 kDa and HA-degrading activity was inhibited to the basal level by *G. thunbergii* extract in a dose-dependent manner. They also studied the expression levels of KIAA1199 mRNA and protein at 24 and 72 hours by treating the cells with 3 mg/ml and 10 mg/ml of the plant extract. They reported that at the highest concentration which was 10mg/ml more inhibition occurred as the mRNA levels were lower compared to the control. The mRNA levels and the protein expression at 24 and 72 hours after the treatment were measured by quantitative real-time PCR and immunoblotting respectively. This approach was different to what was used in the present study which was using the Human CEMIP/KIAA1199 ELISA kit. Another difference was the concentrations, Yoshida et al., used high concentrations of the *G.*

thunbergii extract, whereas in the present study low concentrations of *E.tectorum* were used. Although the methods were not similar, the outcome was the same; the plant extracts were able to inhibit KIAA1199 protein. In another study Yoshida et al., 2019[b] evaluated the inhibitory effects of *Sanguisorba officinalis* root extract on KIAA1199-mediated hyaluronic acid degradation and skin wrinkling. They used the similar technique as above on normal human skin fibroblasts (Detroit 551 cells). The root extract of *Sanguisorba officinalis* inhibited KIA1199-mediated HA degradation in skin fibroblasts by down-regulating KIAA1199 mRNA and protein expression.

i



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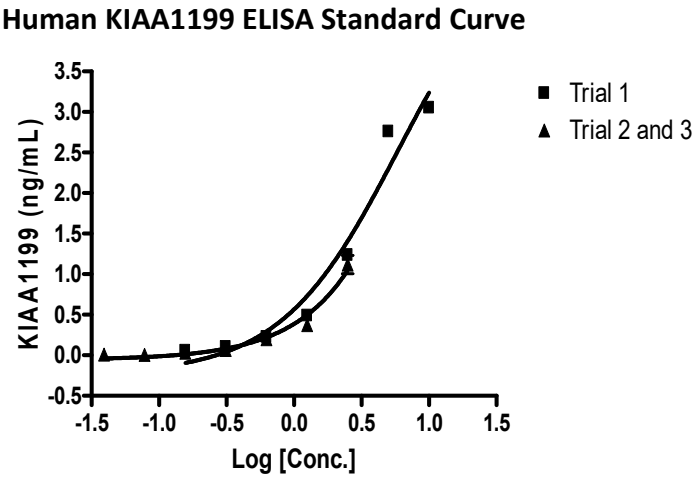

Figure 4.4.3. (i) KIAA1199 protein inhibition by *Elegia tectorum* ethanolic extract at 15, 60 and 240 µg/ml. (ii) ELISA standard curve of KIAA1199 protein. Different letters indicate significant difference. "*" means p< 0.05 significant, "" means p< 0.01 very significant (Tukey's HSD).**

Mutagenicity (Ames test)

The Ames test is typically used for screening for point-inducing mutations. Table 4.4.1 and Figure 4.4.4. shows the results of the mutagenic effect of the ethanol extract of *E. tectorum* on the *S. thyphimurium* TA98 strain. Mortelmans and Zeiger (2000), indicated that positive results from strain TA98 shows frame-shift mutations due to reversion of *Salmonella typhimurium* from His^- to His^+. The plant extract should show a dose-dependent increase in the number of revertant

colonies in order to be considered mutagenic. The number of revertant colonies of the extracts must be greater by a factor of two than that of the negative control (Maron and Ames, 1983). The highest concentration 5000 µg/ml *E.tectrorum* had 131.33 revertant colonies, 500 µg/ml *E.tectrorum* had 121.33 revertant colonies, 50µg/ml had 103.50 revertant colonies. The negative control dH_2O had 100 revertant colonies and the positive control 4-NQO had 463.33 revertant colonies. In this study the extract showed no dose-dependant increase, no revertant colonies were equal to or greater than twice the number of revertant colonies of the negative control. This means that the plant does not have direct mutagenic compounds. According to literature the positive and negative controls were in accordance and within the normal limits (Elgorashi et al., 2003).

There is no literature supporting the non-mutagenic effect of *E.tectorum*. Madikizela et al., (2014), investigated the mutagenic effect of various plant extracts, in which *Pentanisia prunelloides* ethanol extract was found to be non-mutagenic on the TA98 strain. Extract concentration of 5000, 500, 50 µg/m had 20.33, 22.00, 22.33 revertant colonies respectively. The negative control, dH_2O, had 21.00 revertant colonies. The positive control 4-NQO had 133.00 revertant colonies. The ethanol extract of *P. prunelloides*, like *E.tectorum* did not meet the requirements of a mutagenic agent. Therefore, it was concluded to be non-mutagenic. In the same study other ethanol extracts that were tested were *Terminalia phanerophlebia*, *Leonotis intermedia*, *Indigofera arrecta*, all of which were non mutagenic. The study by Eldeen et al., (2005) correspond to the present study, ethanol extracts of *Acacia nilotica, Acacia sieberiana, Albizia adianthifolia, Combretum kraussi, Salix mucronata, Trichilia dregeana* were non-mutagenic on TA98 strain.

Table 4.4.1 Number of revertant colonies of *Salmonella typhimurium* strain TA98 induced by extract of *Elegia tectorum*

Concentration of *E.tectorum*	**Average of revertant colonies**	**± Standard deviation**
5000µg/ml	131.33	10.84
500µg/ml	121.33	26.71

50µg/ml	103.50	14.5
4-NQO (Positive control)	463.33	40.53
dH$_2$O (Neg control)	100	16.87
10% DMSO	100	8.524

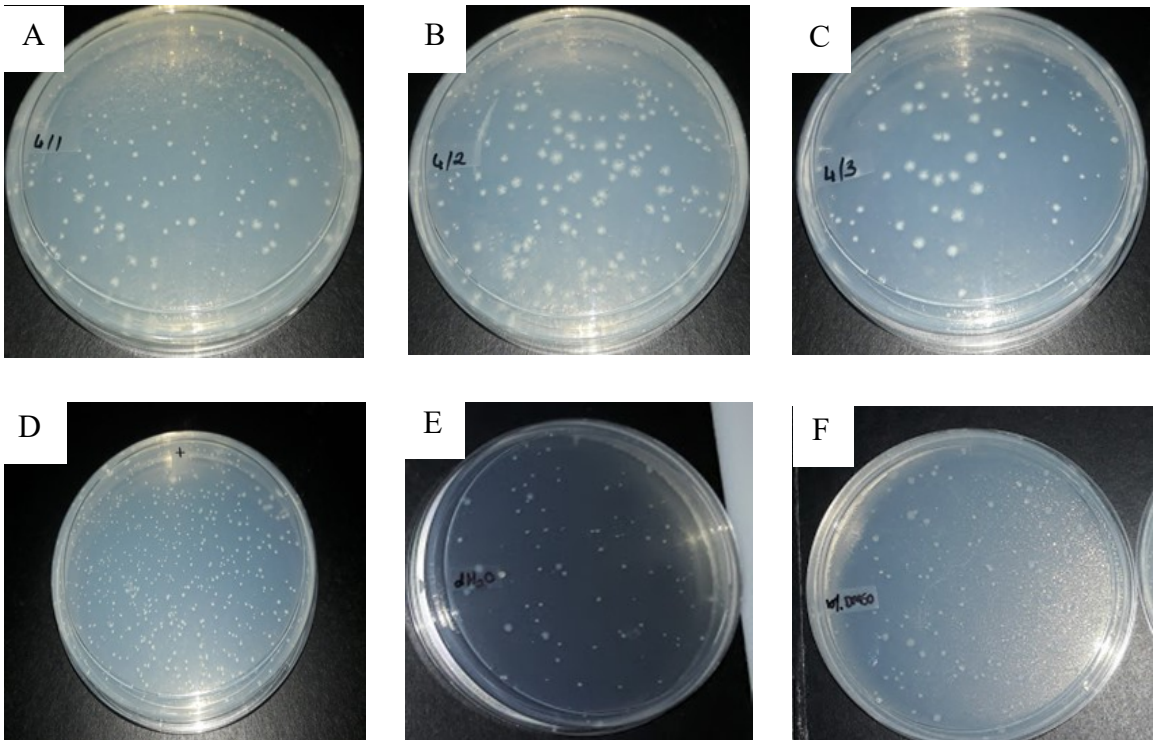

Figure 4.4.4. Plates showing revertant colonies of *Salmonella typhimurium* strain TA98 induced by *Elegia tectorum* ethanolic extract. A - 5000µg/ml, B - 500µg/ml, C - 50µg/ml, D - 4-NQO (positive control), E - dH$_2$O (Negative control), F -10% dimethyl sulfoxide (DMSO).

Molecular docking

Molecular docking was conducted in order to predict the binding mode of the compounds in the *E. tecorum* extract to the elastase enzyme. In the previous section on elastase inhibition, it was shown that the ethanolic extract was the best inhibitor of the elastase enzyme. The concentration required to inhibit 50% of the enzyme (IC_{50}) was 14.58µg/ml. The elastase enzyme was selected for molecular docking.

Binding site prediction

The crystal structure of elastase enzyme (PDB: 1U4G) was co-crystalized with an inhibitor which is bound to the active site (Thayer et al., 1991) (Figure4.4.5). In the process of structure-based drug design, identification and characterization of binding sites is key important (Halgren, 2009). SiteMap from Schrodinger (2017) was used to predict the possible binding site. The smiles of the 57 compounds were submitted to the SwissModel blind docking server in order to identify the most favorable binding site (Lohning et al., 2017). These analyses recognized the same sites as the ideal binding site for six molecules, the same site as the crystal bound ligand.

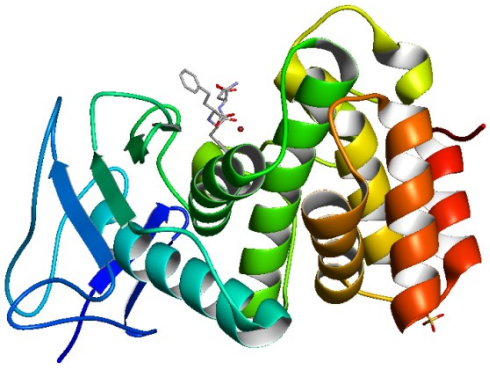

Figure 4.4.5. Representation of the elastase enzyme (PDB: 1U4G)

Molecular docking is used to determine the binding modes, fit and poses of compounds in the binding site of the enzyme (Lohning et al., 2017). Glide score is an empirical scoring function that is used to approximate the ligand binding free energy (Friesner et al., 2006). Six compounds out of 57 were docked successfully using Glide XP (Table 4.4.2), the docking scores range from -6.92 to -5.02. Tight binding or affinity is represented by more negative values. The reference ligand had a score of -11.644. Octadecanoic acid had a score of -6.915, which was the closest to the reference ligand. There is usually correlation made between biological activities and docking scores. However, Glide and other docking programs such as GOLD and SwissDock are used for database improvement and show analysis and ranking instead of predicting binding affinities (Friesner et al., 2006).

The E-model weighs the force field components more significantly (electrostatic and van der Waals energies), this makes it suitable for comparing conformers and less for comparing chemically distinct molecules. Thus, Glide uses E-model to choose the "best" position of a ligand and ranks these best positions against one another with GlideScore (Friesner et al., 2006) The reference ligand had the lowest score (-105.38) which was expected as this showed successful docking. It was followed by 3-(5-Methylfuryl)-N-furamidopropionamide (-58.29) and Hexanedioic acid, bis(2-ethylhexyl) ester (-58.14) (Table3.2). The Glide E-model scores of these two compounds were not close to the reference ligand which could indicate that there was no good “best position” for the molecules to bind as perfect at the reference compound. These compounds in future can be tested for elastase inhibition to determine the effect they have on enzyme inhibition.

Table 4.4.2. Docking scores of GC-MS identified ligands of *Elegia tectorum* docked in the active site of the elastase enzyme model.

Ligand	**Glide XP score**	**Rotatable bonds**	**glide evdw[a]**	**glide ecoul[b]**	**glide energy**	**Glide E-model**
Reference compound	-11.64	14	-35.66	-31.59	-67.25	-105.38
Octadecanoic acid	-6.91	16	-25.33	-10.16	-35.49	-42.36
9,12,15-Octadecatrienoic acid, (Z,Z,Z)	-6.39	13	-26.38	-8.75	-35.13	-40.82
n-Hexadecanoic acid	-6.20	14	-21.82	-10.97	-32.79	-37.318
3-(5-Methylfuryl)-N-furamidopropionamide	-5.21	7	-31.64	-11.58	-43.22	-58.29
Hexanedioic acid, bis(2-ethylhexyl) ester	-5.02	19	-33.76	-10.87	-44.63	-58.14

[a] Van der Waals energy, [b] Coulomb energy

Isolation (Column chromatography)

The crude ethanolic extract was portioned into three fractions namely: ethyl acetate, *n*-butanol and water. These were dried and then tested against the elastase enzyme, Table 4.4.3 shows the

IC_{50} values. The water fraction showed good elastase inhibition with an IC_{50} of 34.44 µg/ml compared to the other fractions, ursolic acid was the positive control and it had an IC_{50} of 4.42 µg/ml. The water fraction was used further for isolation.

Table 4.4.3. Elastase inhibition of fractions obtained by liquid-liquid partitioning of *Elegia tectorum* into three fractions: ethyl acetate, *n*-butanol and water.

***Elegia tectrorum* fractions**	**IC_{50} (µg/ml) ± Standard deviation**
Water	34.44 ± 3.55
n-butanol	63.51 ± 3.52
Ethyl acetate	110.2 6 ± 5.94
Ursolic acid (positive control)	4.42 ± 2.55

The TLC was used to assess the chemical profile of the water fraction (solvent system: ethyl acetate +methanol+water, 6.5+3.5+1.0 ml respectively) . One major compound was observed on the TLC. The water fraction was then subjected to silica gel column chromatography (Figure 4.4.6). A total of 265 fractions were eluted from the silica gel column chromatography based on polarity, the TLC plates of all 265 fractions can be found in the appendix A. The fractions were pooled together based on their TLC profiles in the following manner: fraction 1-12 (82.4 mg), fraction 13-21 (71 mg), fraction 22-62 (541.4 mg), fraction 63-175 (1539.7 mg), fraction176-250 (617.9 mg) and fraction 251-265 (280.9 mg).

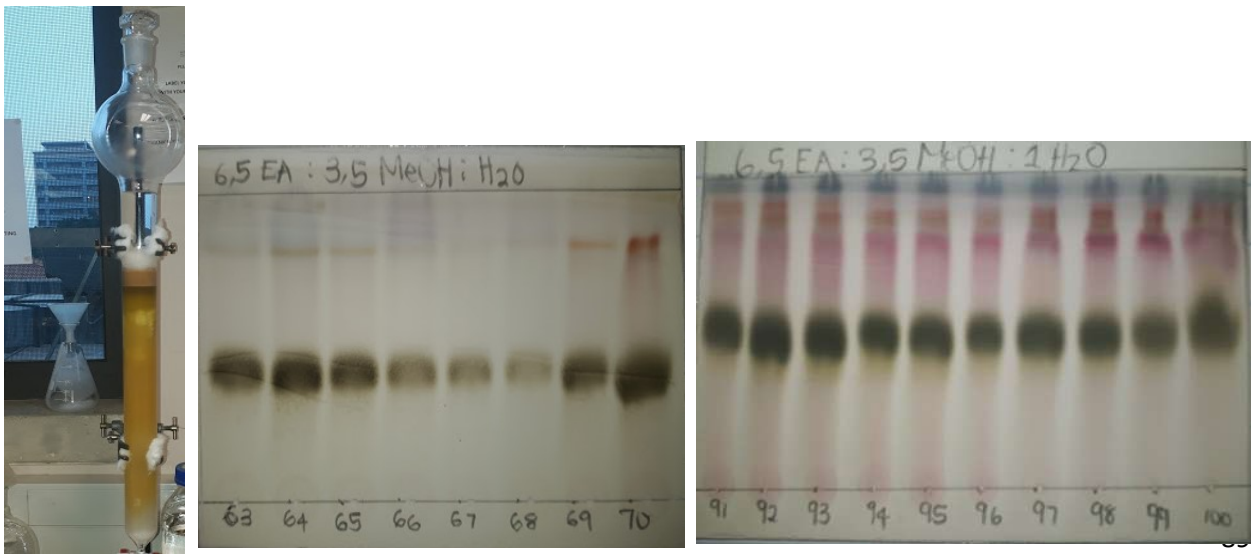

Figure 4.4.6. Silica column chromatography on the left and TLC plate of several fractions isolated

The pooled fractions were tested against elastase, none of the fractions exhibited elastase inhibition (Table 4.4.4). According to Lafi et al., 2018, such observation shows that crude extracts sometimes surpass the efficacy of isolated compounds due to synergistic effects. The compound that was common in most of the fractions was the target compound (green) (Figure 4.4.7 A). As mentioned in experimental section it was purified from the pooled fraction F22-62 by precipitation. The 1D and 2D NMR spectra of this compound indicated that this compound is an oligosaccharide containing two aldose and two ketose sugars (Figure 4.4.7 B). The anomeric proton and carbon NMR signals indicate that both sugars have one alpha and one beta anomer. Tentative structure of the compound is given in (Figure 4.4.7 B). However, the linkage of the sugar residues couldn't be confirmed due to the overlapping of NMR peaks. ^{1}H NMR (400MHz, CD_3OD): δ_H, 3.14-3.76 (26H, m), 4.51 (1H, d, J = 6.2 Hz), 5.14 (1H, d, J = 2.9 Hz). **^{13}C NMR (400MHz, CD_3OD):** δ_C, 62.8 (CH_2), 62.9 (CH_2), 64.2 (CH_2), 64.6 (CH_2), 64.7 (CH_2), 65.9 (CH_2), 69.43 (CH), 71.28 (CH), 71.81 (CH), 71.90 (CH), 71.92 (CH), 73.08 (CH), 73.87 (CH), 74.93 (CH), 76.35 (CH), 76.84 (CH), 77.68 (CH), 78.09 (CH), 78.13 (CH), 83.33 (CH), 94.02 (CH), 98.26 (C), 99.34 (CH), 103.22 (C).

Table 4.4.4. Elastase inhibition of the six pooled fractions.

Fraction	IC_{50} µg/ml ± standard deviation
1	>250
2	>250
3	>250
4	>250
5	>250
6	>250
UA	4.63 ± 1.33

Figure 4.4.7. (A) TLC plates of the pooled fraction of *Elegia tectorum*, the green compound highlighted in black was the compound of interest. (B) Tentative structure of the purified molecule.

Stability of cream formulation and extract

The results for stability of the cream formulation with extract were observed from week 0 and compared to the results obtained after 12 weeks (Table 4.4.5, Figure 4.4.8). Less change was expected for extracts and creams stored at lower temperatures and more change was expected at accelerated temperatures. The initial appearance of the product was pale green/light green and thick due to the plant extract added. The appearance (color and viscocity) did not change after 12 weeks for 4ºC and 25ºC. However, the appearance of the product was runny and yellow for 40ºC and 50ºC after 12 weeks. This may have been a physical change or chemical change caused by the temperature, also, darkening of the products that have extracts is normal (Lall et al., 2017). Initially the product was odorless, and it remained like that for 4ºC and 25ºC. There was a faint odor for the two higher temperatures (40ºC and 50ºC). The initial pH was 5.66 and it dropped slightly to 5.59, 5.36, 5.11 and 5.03 for 4ºC, 25ºC, 40ºC and 50ºC samples, respectively

after 12 weeks. From the results, samples that were stored at a high temperature showed a reduction in pH compared to the samples stored at lower temperature. Viscosity decreased in the values at accelerated temperatures, it increased slightly at 25ºC and increased further at 4ºC. pH and viscosity do not affect the overall performance of the product (Lall et al., 2017). These results indicate that the product should be stored at temperatures below 40 ºC and away from sunlight.

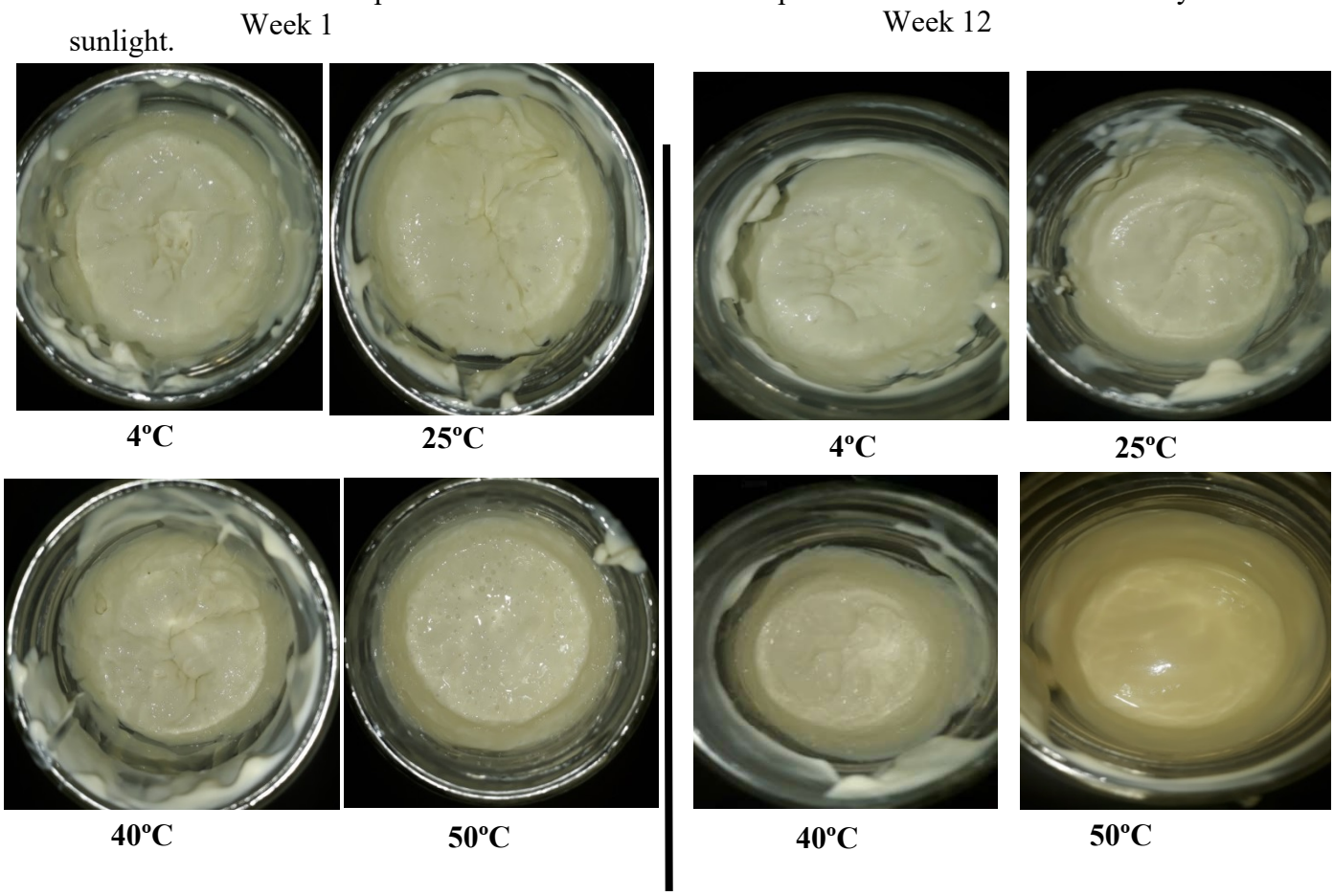

Figure 4.4.8. Cream formulations with ethanolic extract added stored at different temperatures, showing difference between week 1 and week 12.

Table 4.4.5. Stability of the cream formulation with *Elegia tectorum* extract based on appearance, odor, pH and viscosity

		Cream + Extract				
TEST	**STORAGE CONDITIONS**	**STORAGE INTERVALS**				
		1 Week	2 Week	4 Week	8 Week	12 Week
Appearance: Initial result: Pale green/light green	4°C	Complies	Complies	Complies	Complies	Complies
	25°C (RT)	Complies	Complies	Complies	Complies	Complies
	40°C	Complies	Complies	Decrease in viscosity	Decrease in viscosity	More decrease in viscosity and yellow in colour
	50°C	Decrease in viscosity	Decrease in viscosity	More decrease in viscosity	Further decrease in viscosity	Further decrease in viscosity and yellow colour
Odor: Initial result: Odorless	4°C	Complies	Complies	Complies	Complies	Complies
	25°C (RT)	Complies	Complies	Complies	Complies	Complies
	40°C	Complies	Complies	Faint odour	Faint odour	Faint odour
	50° C	Faint odour	Faint odour	Loss of aromatic profile	Loss of aromatic profile	Loss of aromatic profile
PH at ambient temperature 25°C Initial result: 5.66	4°C	5.65	5.60	5.65	5.72	5.59
	25° C(RT)	5.59	5.62	5.52	5.73	5.36
	40°C	5.57	5.57	5.44	5.66	5.11
	50 °C	5.49	5.45	5.32	5.47	5.03
Viscosity at ambient temperature (25°C) Spindle: R4 @ 20RPM Initial result: 7724.74 mPa-s	4°C	9401.26	9826.76	9658.14	9765.2	9660.78
	25°C (RT)	7717.4	7722.18	8380.7	8594.72	8938.4
	40°C	7197.52	7371.94	5653.12	4603.48	3842.5
	50 °C	5381.36	3321.44	1979.82	1311.82	1199.5

The results for the stability of the ethanolic extract were taken at week 0 and were compared to those after week 12. More change was expected for extracts that were stored at accelerated temperatures and less change was expected for extracts that were at lower temperatures. There was sedimentation of the extracts and change in colour for the extracts that were stored at high temperatures after 1 week (Figure 4.4.9). The extracts stored at 4ºC and 25ºC did not change in appearance after 1 week. The TLC plate in Figure 4.9 showed no change in the compounds that were present in the plant after 1 week. There were more changes observed after 12 weeks, the extracts stored at higher temperatures had more sediments and the colour changed to being brown. The extract stored at 25ºC also had few sediments but there was no colour change and the extract stored at 4ºC did not change (Figure 4.4.10). The TLC plate in Figure 4.4.10 shows the compounds that were present in the extracts after 12 weeks, for the two extracts that were stored at lower temperatures there was a red compound at the bottom that was dominant and not fully present in the extracts stored at accelerated temperatures. There was more of the green compound in the middle in the extracts that were stored at accelerated temperatures compared to the extracts stored at lower temperatures.

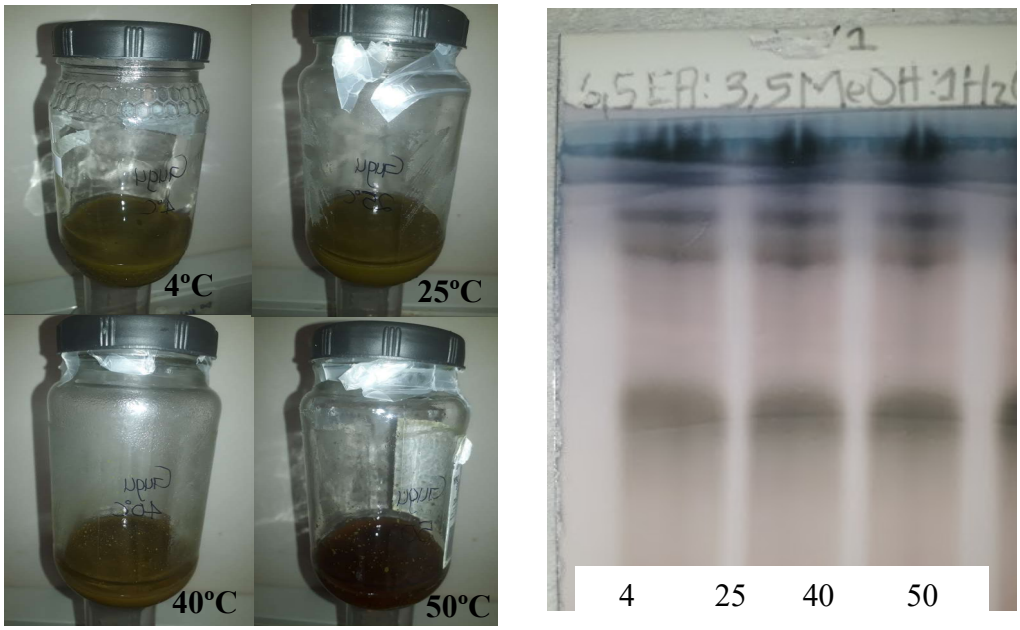

Figure 4.4.9. *Elegia tectorum* ethanolic extract stored at different temperatures and the TLC plate showing the compounds profile in week 1.

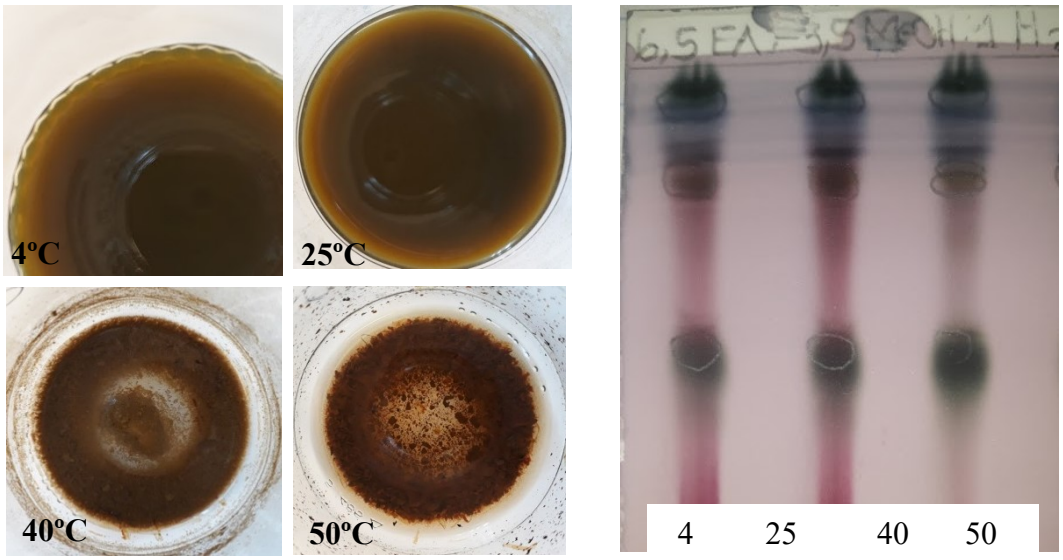

Figure 4.4.10. *Elegia tectorum* extract stored at different temperatures and the TLC plate showing the compounds present in week 12.

After 12 weeks elastase inhibition assay was performed to determine if there are changes in activity due to exposure to different temperatures (Figure 4.4.11). The original IC_{50} value of the ethanolic extract was 14.58 µg/ml. The extracts that were stored at 4ºC and 25ºC had good elastase inhibition with IC_{50} of 14.39 µg/ml and 28.88 µg/ml respectively. At 25˚C there was a slight reduction in activity although the IC_{50} was good as it indicated inhibition. High temperature resulted in more reduction in activity of the extract, 50ºC had an IC_{50} of 92.08 µg/ml and 40ºC had an IC_{50} of 121.20 µg/ml. Ursolic was the positive control with an IC_{50} of 4.23µg/ml. These results confirmed that extract was stable at 4˚C and 25˚C. The lower temperatures were able to preserve the activity of the compounds in the extract responsible for the biological activity. During production of the formulation, these temperatures can be recommended for storage of the extract. No metal toxicity was detected in the plant extract

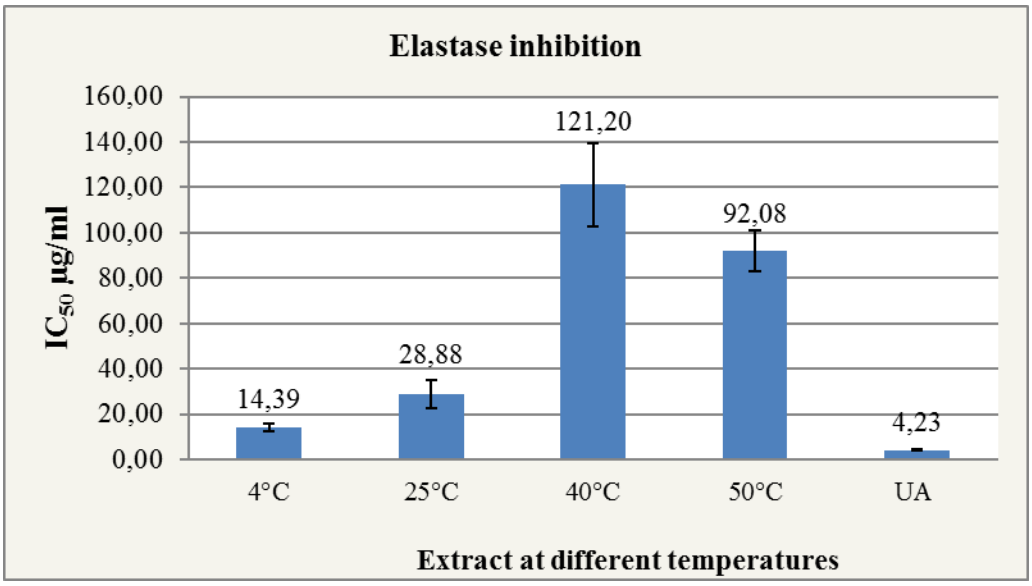

Figure 4.4.11. Elastase inhibition of the ethanolic extract of *Elegia tectorum* stored at different temperatures.

Irritancy patch test

A principle test which shows irritant contact dermatitis is known as the irritancy patch testing. An irritant is any substance that result in inflammation or irritant reaction when applied in high concentration for a long period of time in almost all individuals but does not have effect on the immune system (Lewis, 2014). The visual irritancy test results Table 4.4.6 showed that the positive control, sodium lauryl sulphate solution (1%) was highly irritating to the skin with high irritancy mean score of 1.25, while the negative control, demineralized water was non-irritating to the skin with low irritancy mean score of 0.30. *Elegia tectorum* ethanolic extract, neat was found to be a mild irritant with irritancy mean score of 0.33. The results were interpreted as follows: Non-irritant: Mean Score (average plus standard deviation) falls below that of the negative control, test substances performed better or similar to that of demineralized water. Mild Irritant: Mean Score (average plus standard deviation) falls above that of negative control, but was lower than that of the positive control (Appendix B1). A percentage increase in compatibility potential relative to negative control is given. Irritant: Mean Score (average plus standard deviation) falls above that of the positive control, test substances performed worse than

the positive control. The ethanolic extract of *E.tectorum* was found to be a mild irritant, these results could be because it was applied neat, and ethanol can cause a reaction on the skin. There are no previous skin irritancy studies that have been conducted on *E. tectorum*. Extract in the aqueous cream was not found to be irritant (Appendix B2).

Table 4.4.6. Irritancy score mean value when 1% sodium lauryl sulphate (positive control), demineralized water (negative control) and *Elegia tectorum* ethanolic extract was applied on the inner forearm area observed at 24, 48 and 72 hours (*n*=20).

	Average value	Mean score	Number of subjects with reactions after 48 hours	Skin compatibility Potential % (TP-NC)/(PCNC)	Skin Compatibility
Positive control sodium lauryl sulphate solution (1%)	0.59	1.25	8	100	Irritant
ET ethanol extract (neat)	0.12	0.33	0	3.06	Mild irritant
Negative control-demineralized water	0.13	0.30	0	0.00	Non-irritant

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Chapter 5
Conclusion and future prospects

CHAPTER 5

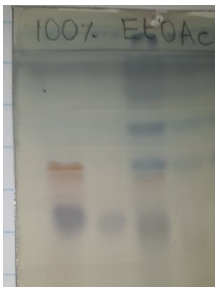
5.1. Conclusion

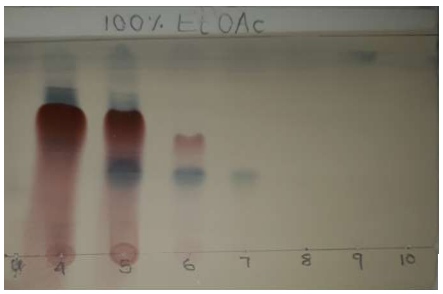
Based on the results, the research question which was can a South African wetland plant and its isolated compounds reduce wrinkle formation and retain skin hydration, was answered. The ethanolic extract had good elastase inhibition. It had no toxicity on HT-29 cells, and it was able to inhibit the KIAA1199 protein which is responsible for degrading hyraluronic acid. By inhibiting KIA1199, less hyaluronic acid will be degraded; this will improve the hydration of the skin. The ethanolic extract does not cause mutations and the compounds in the plant work well together synergistically rather than when they are isolated. An irritancy test was conducted on 20 healthy volunteers to see if the extract resulted in an adverse reaction on the skin. The extract, when applied neat, was found to be a mild irritant but in aqueous cream (10%), it was found to be non-irritant. During efficacy study, it was found that at 10% of extract in aqueous cream; was effective in decreasing the wrinkles from fourteen 14 (D14) up to twenty-eight (D28) of consecutive use (twice a day) when compared to a placebo control. The study therefore, resulted in a valuable prototype for ageing problems.

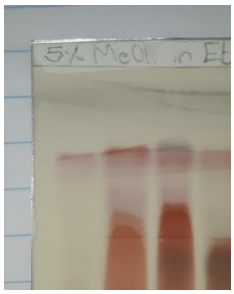
5.2. Future prospects

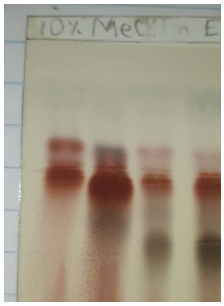
For future studies, the plant extract can be put in a formulation and clinical trials can be conducted, should the plant be successful in all the phases of the clinical trials, then a medicinal anti-aging cream will have the potential to be marketed. More plants in the Restionaceae family can be investigated to determine if they can reduce wrinkle formation as there is not much information on the plants in this family. The plants in this family can also be compared amongst each other to see if there are compounds that are similar which can have the same biological effect. *Elegia tectorum* extract should be further investigated to identify its effect on other skin related conditions and it can be combined with other plants to understand whether it enhances or reduces activity.

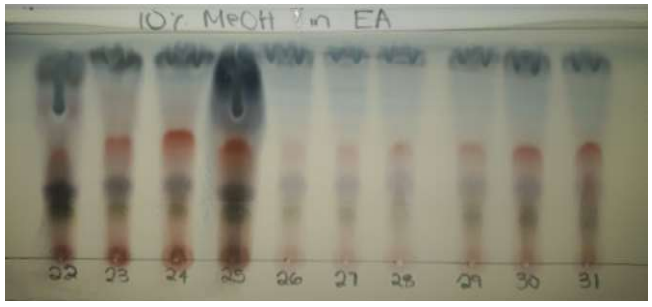
Appendix A: Fractions obtained during column chromatography of *Elegia Tectorum*

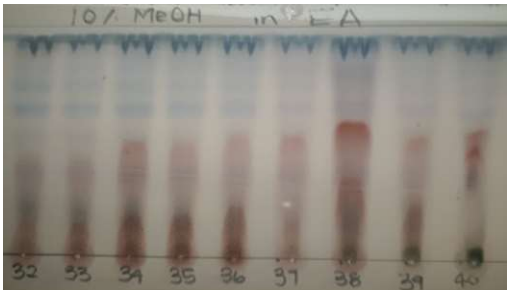


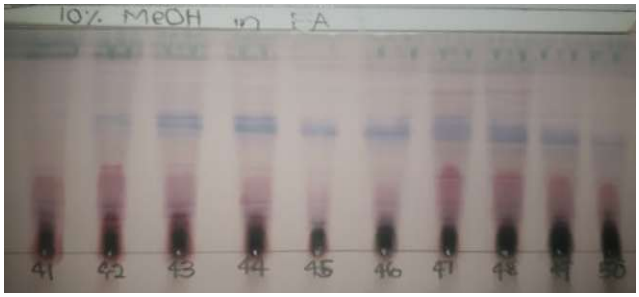


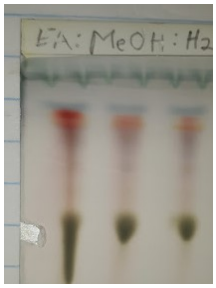


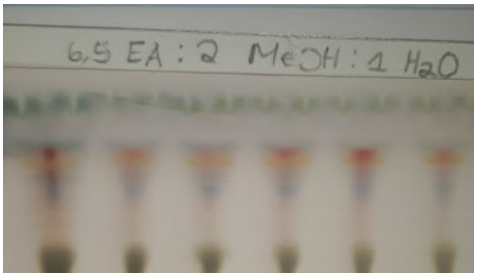


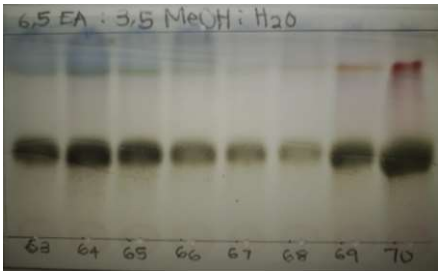


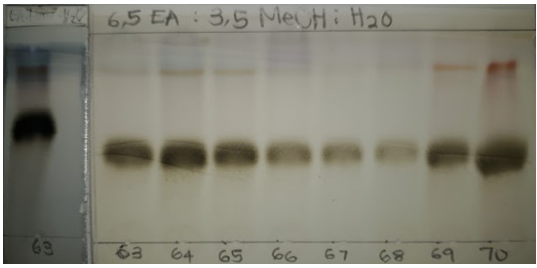


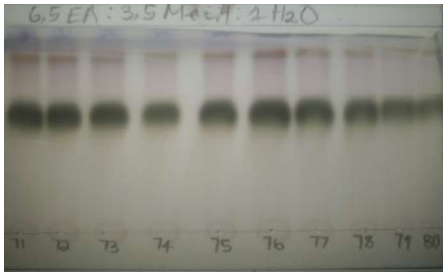


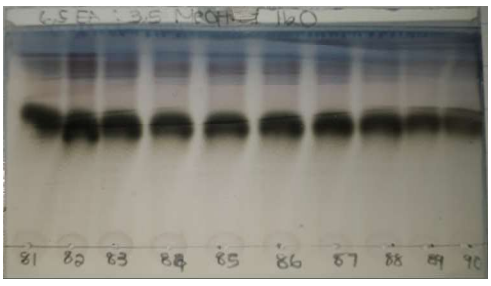


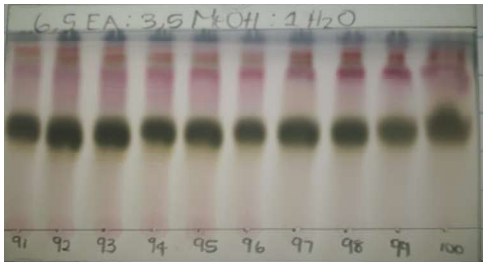




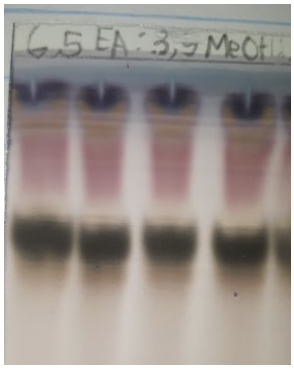


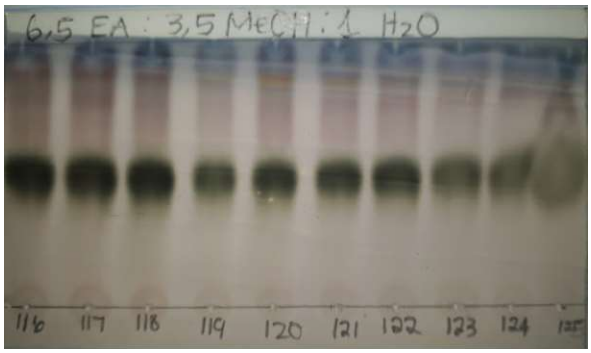


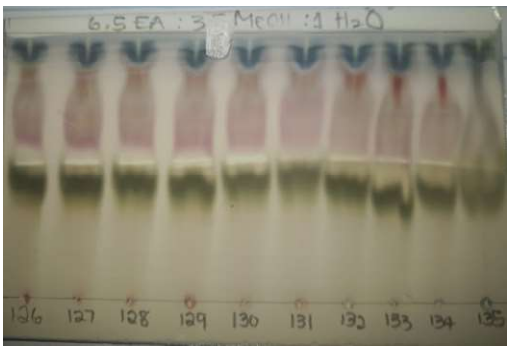


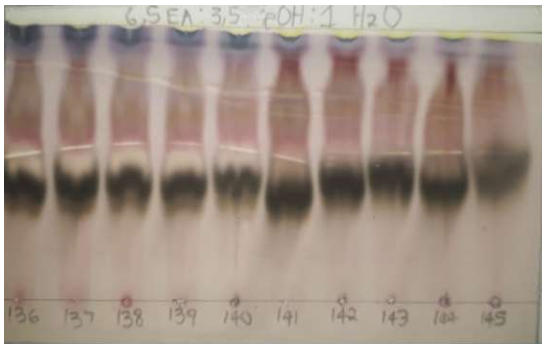


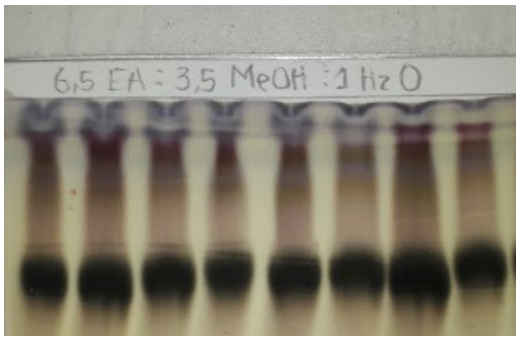

6,5 EA : 3,5 MeOH : 1 H2O
101 102 103 104 105 106 107 108 109 110

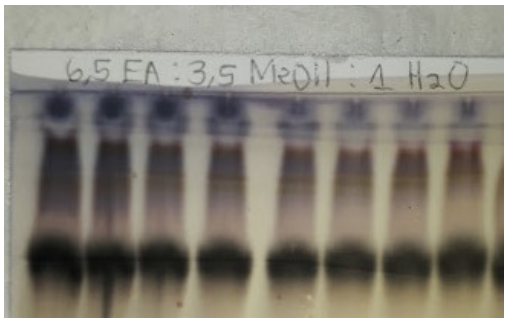

6,5 EA : 3,5 MeOH

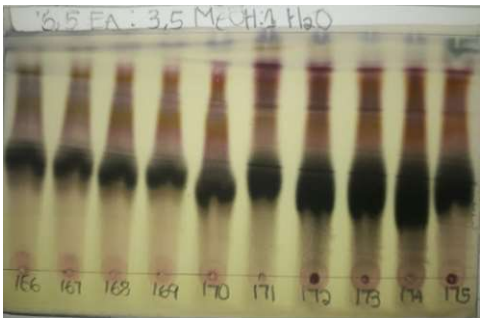

6,5 EA : 3,5 MeOH : 1 H2O
116 117 118 119 120 121 122 123 124

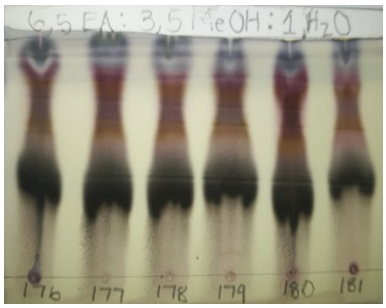

6,5 EA : 3,5 MeOH : 1 H2O
126 127 128 129 130 131 132 133 134 135

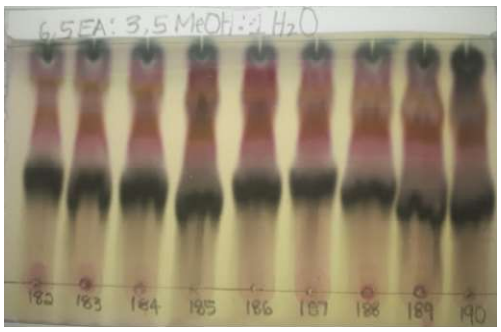

6,5 EA : 3,5 MeOH : 1 H2O
136 137 138 139 140 141 142 143 144 145

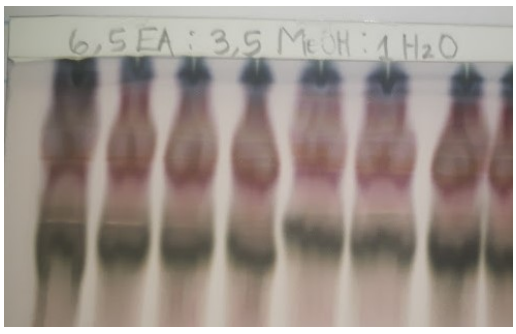

6,5 EA : 3,5 MeOH : 1 H2O

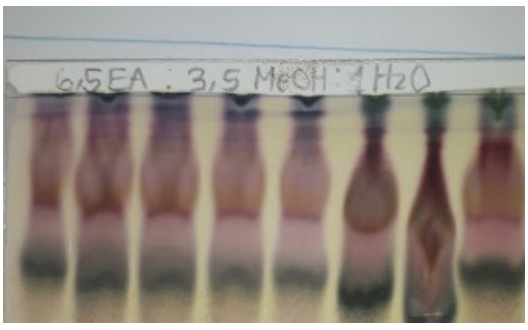

6,5 EA : 3,5 MeOH : 1 H2O

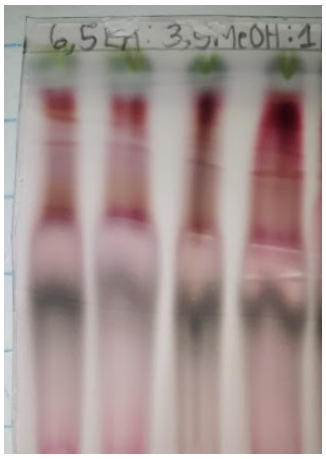

6,5 EA : 3,5 MeOH : 1 H2O
166 167 168 169 170 171 172 173 174 175

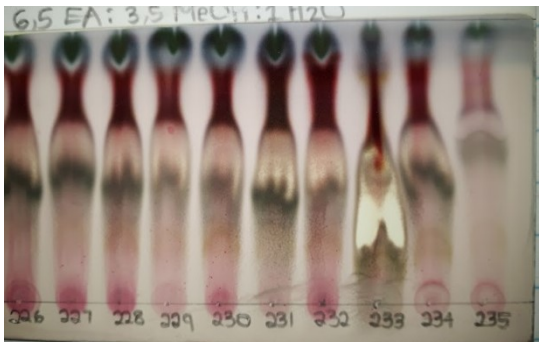

6,5 EA : 3,5 MeOH : 1 H2O
176 177 178 179 180 181

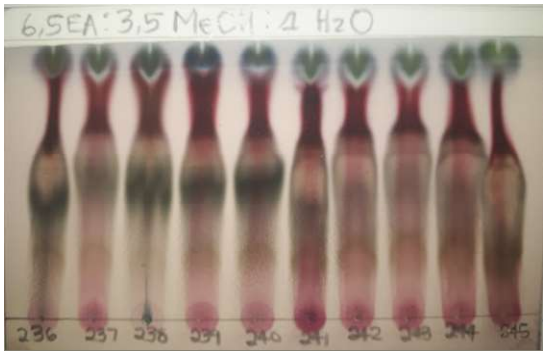

6,5 EA : 3,5 MeOH : 1 H2O
182 183 184 185 186 187 188 189 190

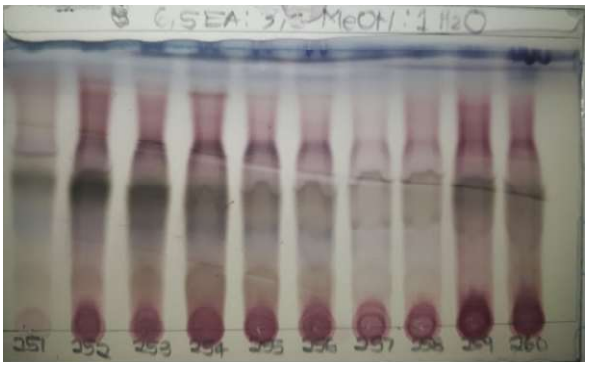

6,5 EA : 3,5 MeOH : 1 H2O

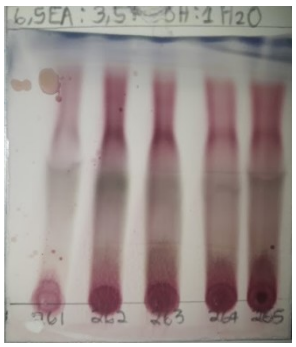

6,5 EA : 3,5 MeOH : 1 H2O











Appendix B1: Irritancy study of *Elegia tectorum* neat extract

FUTURE COSMETICS CC

From Concept to Product

ATT :NAMRITA LALL

UNIVERSITY OF PRETORIA
ROOM 3-32 NATURAL SCIENCE
HATFIELD (MAIN) CAMPUS
UNIVERSITY OF PRETORIA
LYNNWOOD ROAD
PRETORIA

2017-12-07

Re: Report on the Skin compatibility Study Performed: FCSS268

Herewith the report on the primary skin compatibility study for the following test products:

FCSS268	/PC	POSITIVE CONTROL
FCSS268	/5866	ET UP SAMPLE ANTI - WRINKLE (10%) ; IRRITANCY (NEAT)
FCSS268	/5868	PS UP SAMPLE IRRITANCY (PS NEAT)
FCSS268	/NC	NEGATIVE CONTROL - DEMINERALISED WATER

Attached please find the report, calculations and invoice. If all is not included, please contact us within 14 days of receipt of results.

Please do not hesitate to contact me.

Kind Regards

Heibrie Le Roux
(Managing Member)

Member:Heibrie Le Roux Registration No:2001/055088/23
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Tel:+27-128110621 Fax:+27-128110510 Mobile:+27-829613509 E-mail:heibrie@futurecosmetics.co.za

FUTURE COSMETICS CC

From Concept to Product

2017-12-07

REPORT ON 24 HOURS OCCLUSIVE COMPATIBILITY PATCH TESTING PERFORMED

HUMAN PATCH TESTING

STUDY REFERENCE NUMBER: FCSS268

Test on primary skin compatibility on human subjects.

Responsible for study:	Heibrie Le Roux Managing Member

Products tested:	FCSS268	/PC	POSITIVE CONTROL
	FCSS268	/5866	ET UP SAMPLE ANTI - WRINKLE (10%) ; IRRITANCY (NEAT)
	FCSS268	/5868	PS UP SAMPLE IRRITANCY (PS NEAT)
	FCSS268	/NC	NEGATIVE CONTROL - DEMINERALISED WATER

Customer:	UNIVERSITY OF PRETORIA ROOM 3-32 NATURAL SCIENCE HATFIELD (MAIN) CAMPUS UNIVERSITY OF PRETORIA LYNNWOOD ROAD PRETORIA
Concentration of products:	Neat

The following report is an accurate account of the test method as described in the protocol FCSOP100 and the results obtained during the course of the study, which was performed within the month prior to the date of this report.

A Sensitive population was tested and identified before the initiation on this study as per FCSOP100.The test subject's volar forearm was cleansed with alcohol and tape stripped in order to ensure very sensitive skin before conducting a human patch test

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The objective of the study was to detect primary skin compatibility potential of the tested products on the skin of human test subjects. Patch testing represents a relatively safe and reasonable reliable method for identifying irritants. A positive reaction to a correctly applied patch test could prove that the person experienced a contact irritation from the substance tested.

Test Protocol:

Materials:
Test Products
Cotton Buds
Demineralised Water
8mm Finn Chambers
Finn Chamber Templates
Black Marker Pens
Positive Displacement Pipette
Sodium Lauryl Sulphate Solution (1%) as a Positive Control
Demineralised Water as a Negative Control

Camera:

Sony Cyber-shot Digital Camera (1.3 mega pixel) and Sandisk memory stick.

Products, applicators and sequence:
8mm Finn Chambers on micropore tape was used to allow for occlusive testing.
Where necessary, transpore tape was applied to secure the strips in a manner that ensured even pressure dispersion across the test area.
1CC Syringes were used to apply the controls and products.

Method:
Twenty (20) subjects between the ages of 21 and 64 were recruited. (See Appendix A: Subject Demographics). The procedure of patch testing was explained to them verbally and each subject signed a form of consent and medical history (See Appendix D : Consernt Form and Medical history). Personal details and each subject's assessment of their own skin type were recorded.

Visual assessments of the test sub-sites were made at 24, 48 and 72 hours. The controls and products were applied to the inner forearm at 0 hours. The finn chambers covered the patch areas for the first 24 hours after which they were removed and the subject could clean the area by any normal mean.

Colour photographs were taken at each time interval to serve as a recording of the observations.

The controls and product were applied to the inner forearm according to a rotating position sequence in order to avoid position and recording bias (See Appendix C: Test Substance Sequence).

The following rating system was used to classify the reactions:

0 = No response
0.5 = Minimal/doubtful response
1 = Mild erythema, spotty or diffuse
2 = Definate erythema, Uniform redness, itching or burning response. Swelling may occur
3 = Strong and severe uniform redness, swelling or spreading beyond the area of the disc may occur
4 = Fiery redness, oedema, papules, bullae

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Dermatological Criteria:

Positive reactions of an allergenic nature are profound and clear red, commonly with minute papules or vesicles, which in severe reactions coalesce into bullae. The diffuse and spread beyond the area of the disc. As with contact dermatitis, there is usually itching and burning sensations.

Once a reaction has developed, the positive reactions persist for several days. A reaction noted at 2 days (48 hours) and negative at 3 days (72 hours) are often of an irritant nature.

A positive reaction on skin sensitisation has a very similar reaction to that of an irritant and normally occurs upon the second application (24 hours) if a sensitising species are present, provided about 24 hours separate successive applications have been performed therefore appearing at 48 hours; although with some substances the relationship is not clear cut.

The classification of results is interpreted as follows:

Non-irritant = Mean Score (Average plus standard deviation) falls below that of negative control, test substances performed better or similar to that of demineralised water.

Mild Irritant = Mean Score (Average plus standard deviation) falls above that of negative control, but was lower than that of positive control. A percentage increase in compatibility potential relative to negative control is given.

Irritant = Mean Score (Average plus standard deviation) falls above that of positive control, test substances performed worse than the positive control.

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Results and Conclusions:

A Summary of the results is given in Attachment E.

TEST RESULTS VALUES AFTER SEVENTY-TWO (72) HOURS

PRODUCT NUMBER	TEST REFERENCE	PRODUCT REFERENCE	TEST PRODUCT NAME:	Average Value	Mean Score	Number of subjects with reactions after 48 hrs	Skin Compatibility Potential % (TP-NC)/(PC-NC)	Skin Compatibility
1	FCSS268	/PC	POSITIVE CONTROL	0.59	1.25	8	100.00	Irritant
4	FCSS268	/5866	ET UP SAMPLE ANTI - WRINKLE (10%) ; IRRITANCY (NEAT)	0.12	0.33	0	3.06	Mild Irritant
5	FCSS268	/5868	PS UP SAMPLE IRRITANCY (PS NEAT)	0.23	0.48	0	18.66	Mild Irritant
11	FCSS268	/NC	NEGATIVE CONTROL - DEMINERALISED WATER	0.13	0.30	0	0.00	Non Irritant

Kind regards

HEIBRIE LE ROUX
(MANAGING MEMBER)

Member:Heibrie Le Roux Registration No:2001/055088/23
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FUTURE COSMETICS CC

From Concept to Product

Data Sheet of Skin Compatibility Study

INITIATION DATE: 2017/11/27
COMPLETION DATE: 2017/11/30
TEST REF: FCS3268

Product 1	FCS3268 /PC									POSITIVE CONTROL												MEAN
Volunteer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	AVG	STDEV	SCORE
24 hours ev 1	1.5	0	1	0	1.5	0	1	1.5	0	0.5	2	2	0	0	0.5	1.5	0	1.5	1	0	0.85	0.82	1.67
24 hours ev 2	1	0.5	1.5	0	1.5	0	1	1.5	0	0.5	2.5	2.5	0	0	0.5	2	0	2	1.5	0			
48 hours ev 1	0.5	0.5	1	0	1	0	0.5	0.5	0	0	1.5	1.5	0	0	0	1	0	0.5	1.5	0	0.59	0.66	1.26
48 hours ev 2	0.5	0.5	1.5	0	1.5	0	0.5	1	0	0	2	2	0	0	0	1.5	0	1.5	1	0			
72 hours ev 1	0	0	1	0	1	0	0	0	0	0	0.5	1	0	0	0	0.5	0	0.5	0.5	0	0.34	0.50	0.84
72 hours ev 2	0	0	1.5	0	1	0	0	0.5	0	0	1	1.5	0	0	0	1.5	0	1	0.5	0			
Average value for product over 72 hours																					0.59	0.66	1.26
Percentage Compatibility Potential																					100.00		
Number of test subject experiencing reaction after 48 hours																					8		
Subjects	-	-	3	-	5	-	-	8	-	-	11	12	-	-	-	16	-	18	19	-			
Range of reactions observed	a mild, spotty or diffused redness												to		a definate uniform redness								
Compatibility Classification	Irritant x												Non Irritant							Mild Irritant			

FUTURE COSMETICS CC

From Concept to Product

Data Sheet of Skin Compatibility Study

INITIATION DATE: 2017/11/27
COMPLETION DATE: 2017/11/30
TEST REF: FCS3268

Table 1: Summary of observations of products and two controls on 20 volunteers

Product 4	FCS3268 /6866									ET UP SAMPLE ANTI - WRINKLE (10%) ; Compatibility (NEAT)												MEAN
Volunteer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	AVG	STDEV	SCORE
24 hours ev 1	0.5	0.5	0	0	0	0	0	0	1	0	0	0	0.5	0	1	0.5	0.5	0	0	0	0.23	0.34	0.58
24 hours ev 2	0.5	0.5	0	0	0	0.5	0	0	1	0	0	0	0	0	1	0.5	0	0.5	0	0			
48 hours ev 1	0	0	0	0	0	0	0	0.5	0.5	0	0	0	0	0	0.5	0	0.5	0.5	0	0	0.11	0.21	0.32
48 hours ev 2	0	0	0	0	0	0.5	0	0	0.5	0	0	0	0	0	0.5	0	0	0.5	0	0			
72 hours ev 1	0	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01	0.08	0.09
72 hours ev 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Average value for product over 72 hours																					0.12	0.21	0.33
Percentage Compatibility Potential																					3.06		
Number of test subject experiencing reaction after 48 hours																					0		
Subjects	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Range of reactions observed	No reaction												to		no reaction								
Compatibility Classification	Irritant												Non Irritant							Mild Irritant x			

FUTURE COSMETICS CC

From Concept to Product

Data Sheet of Skin Compatibility Study

INITIATION DATE: 2017/11/27
COMPLETION DATE: 2017/11/30
TEST REF: FCS3268

Table 1: Summary of observations of products and controls on 20 volunteers

Product 6	FCS3268 /6868									PS UP SAMPLE Compatibility (PS NEAT)												MEAN
Volunteer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	AVG	STDEV	SCORE
24 hours ev 1	0	1	0	1	1.5	1	1	0	0	0.5	0	0	1	0	1	0	1	1	0.5	0.5	0.63	0.62	1.04
24 hours ev 2	0	1	0	1	1	1	1	0	0	0.5	0	0	1	0	1.5	0	1	1	0	0			
48 hours ev 1	0	0.5	0	0	0.5	0.5	0.5	0	0	0	0	0	0.5	0	0.5	0	0	0	0	0	0.16	0.23	0.38
48 hours ev 2	0	0.5	0	0	0.5	0.5	0.5	0	0	0	0	0	0.5	0	0.5	0	0	0	0	0			
72 hours ev 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00	0.00	0.00
72 hours ev 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Average value for product over 72 hours																					0.23	0.26	0.48
Percentage Compatibility Potential																					18.86		
Number of test subject experiencing reaction after 48 hours																					0		
Subjects	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Range of reactions observed	No reaction												to		no reaction								
Compatibility Classification	Irritant												Non Irritant							Mild Irritant x			

FUTURE COSMETIC

From Concept to Product

Data Sheet of Skin Compatibility Study

INITIATION DATE: 2017/11/27

COMPLETION DATE: 2017/11/30

TEST REF: FCS8288

Table 1: Summary of observations of products and controls on 20 volunteers

Product 11	FCS8288 /NC							NEGATIVE CONTROL - DEMINERALISED WA							
Volunteer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
24 hours ev 1	0.5	0.5	1	0.5	0.5	0.5	0	0	0	0	0.5	0.5	0	0	1
24 hours ev 2	0.5	0.5	1	0.5	0.5	0.5	0	0	0	0	0.5	0.5	0	0	1
48 hours ev 1	0.5	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0.5
48 hours ev 2	0.5	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0.5
72 hours ev 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72 hours ev 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Average value for product over 72 hours															
Percentage Compatibility Potential															
Number of test subject experiencing reaction after 48 hours															
Subjects	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Range of reactions observed	No reaction												to		
Compatibility Classification	Irritant											Non Irritant		x	

FUTURE COSMETI

From Concept to Product

APPENDIX A:
SUBJECT DEMOGRAPHICS (FCSOP

INITIATION DATE:	2017/11/27
COMPLETION DATE:	2017/11/30
STUDY NUMBER:	FCSS268

STUDY AGE RANGE :	**21 to 64**
TOTAL QUANTITY OF TEST SUBJECTS (n)	**20**
AVERAGE POPULATION AGE:	46.3
YOUNGEST TEST SUBJECT:	21
OLDEST TEST SUBJECT:	64
QUANTITY OF SUBJECTS PER AGE CATEGORY :	
UNDER 18: n =	0
18 to 20: n =	0
21 to 30: n =	4
31 to 40: n =	2
41 to 50: n =	4
51 to 71: n =	10
STUDY GENDER INFORMATION:	
FEMALE	80.00%
MALE	20.00%
STUDY ETHNICITY INFORMATION	
NEGROID	0%
CAUCASIAN	100%

STUDY NUMBER	SUBJECT NUMBER	SKIN TYPE (Fritzpatrick)	AGE
FCSS268	/01	II	59
FCSS268	/02	I	54
FCSS268	/03	III	41
FCSS268	/04	III	25
FCSS268	/05	II	46
FCSS268	/06	I	25
FCSS268	/07	I	25
FCSS268	/08	I	64
FCSS268	/09	II	42
FCSS268	/10	I	64
FCSS268	/11	I	60
FCSS268	/12	II	21
FCSS268	/13	II	60

FUTURE COSMETICS

From Concept to Product

APPENDIX B:
TESTING CONDITIONS (REF:F

INITIATION DATE: 2017/11/27
COMPLETION DATE: 2017/11/30
STUDY NUMBER: FCSS268

DISTRIBUTION OF TESTING CONDITIONS OF						
			TEMPERATURE (°C)	MEAN	HUMIDITY (	
			22.6	BASELINE	50.8	
			22.9	T 24HRS	48.6	
			23.7	T 48HRS	48.0	
			25.7	T 72HRS	52.8	
			23.7	TOTAL	49.8	
			TEMPERATURE RANGE	AMBIENT	HUMIDITY F	
		20.6 to	26.7	RANGE	45	
			20.6	MAXIMUM	45.0	
			26.7	MINIMUM	55.0	
TEMPERATURE (Degrees Celsius)				SUBJECT		
BASELINE	T 24 HRS	T 48 HRS	T 72 HRS	REF:	BASELINE	
20.6	21.1	21.9	24.9	FCSS268 /01	50.8	
20.6	21.5	21.9	25.1	FCSS268 /02	50.8	
20.6	21.9	22.0	26.6	FCSS268 /03	50.8	
20.9	21.5	22.1	25.6	FCSS268 /04	50.9	
20.9	21.8	22.3	24.9	FCSS268 /06	50.9	
20.9	22.5	23.6	25.6	FCSS268 /08	50.9	
21.2	22.5	23.8	25.6	FCSS268 /07	52.0	
21.2	22.8	23.7	25.1	FCSS268 /08	52.0	
21.2	22.3	22.6	26.6	FCSS268 /09	52.0	
22.0	23.0	23.4	22.9	FCSS268 /10	51.4	
22.1	23.0	24.2	22.6	FCSS268 /11	51.4	
22.1	22.9	21.9	26.7	FCSS268 /12	51.4	
22.4	23.0	24.1	26.7	FCSS268 /13	53.4	
24.8	23.2	25.5	26.4	FCSS268 /14	48.6	
24.8	23.6	25.1	26.2	FCSS268 /16	48.9	
24.9	23.8	24.7	26.3	FCSS268 /18	49.0	
25.0	24.6	24.9	26.0	FCSS268 /17	50.0	
25.0	24.4	25.0	26.5	FCSS268 /18	50.0	

FUTURE COSME

From Concept to Product

APPENDIX C: TEST SUBSTANCE SEQUENCE

INITIATION DATE: 2017/10/02
COMPLETION DATE: 2017/10/06
STUDY NUMBER: FCSS307

PRODUCT REFERENCE	PRODUCT NAME:	PRODUCT NUMBER
/PC	POSITIVE CONTROL	1
/5866	ET UP SAMPLE ANTI - WRINKLE (10%) ; IRRITANCY (NEAT)	4
/5868	PS UP SAMPLE IRRITANCY (PS NEAT)	5
/NC	NEGATIVE CONTROL - DEMINERALISED WATER	11

TEST SITE:	RIGHT VOLAR FOREAM
APPLICATION AMOUNT:	2mg/cm2
PATCH SIZE:	8mm Finn Chambers

ADDITIONAL INTRUCTIONS
TAPE STRIPPING

SUBJECT NUMBER	RIGHT ARM														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	
/01	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1
/02	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1
/03	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1
/04	21	1	2	3	4	5	6	7	8	9	10	11	12	13	1
/05	21	1	2	3	4	5	6	7	8	9	10	11	12	13	1
/06	21	1	2	3	4	5	6	7	8	9	10	11	12	13	1
/07	20	21	1	2	3	4	5	6	7	8	9	10	11	12	1
/08	20	21	1	2	3	4	5	6	7	8	9	10	11	12	1
/09	20	21	1	2	3	4	5	6	7	8	9	10	11	12	1
/10	19	20	21	1	2	3	4	5	6	7	8	9	10	11	1
/11	19	20	21	1	2	3	4	5	6	7	8	9	10	11	1
/12	19	20	21	1	2	3	4	5	6	7	8	9	10	11	1
/13	18	19	20	21	1	2	3	4	5	6	7	8	9	10	1
/14	18	19	20	21	1	2	3	4	5	6	7	8	9	10	1
/15	18	19	20	21	1	2	3	4	5	6	7	8	9	10	1

FUTURE COSMETICS CC

From Concept to Product

APPENDIX D: CONSENT FORM (PATCH TESTING) FCSOP100/05	FCSS268	SUBJECT NO:	NAM

1. INTRODUCTION: Before agreeing to participate in this study, it is important that you read & understand the following explanation and procedures. Y guarantees or assurances can be given to the results of this study.

2. BACKGROUND: You have been invited to participate in this study designed to evaluate the performance of one/ more products to be tested for irrit in good health and willing to follow study guidelines/rules **(including medication restrictions & avoidance of water) as well as not covering the te** You are not allowed to apply any products, exercise or wet the test sites. In addition if you have any reactions, allergies to cosmetics/ preservatives/fra interfere with the study or use any medication that might affect results or if you are pregnant, you cannot participate in this study. The minimum amour

3. RULES TO COMPLY WITH:

You will be asked to abandon the study without compensation if you have wiped or washed your arm during the first 24 hours. *Please sign i*
I am not currently busy with any other study or will not apply for any others study during this study duration. *Please sign if agree*
I agree to refrain from using any topical products on my arm, or to wash my arm for the first 24 hours. *Please sign if agree*

4. DURATION OF EVALUATION AND PROCEDURES: **Day 1**: You will be asked to read and sign the consent form and your medical history will study. If you meet the inclusion criteria, you must sign an attendance register. The test sites will be examined for hair,bruises,scarring etc. If you qualif a series of products will be applied. **Day 2, 3, 4 and 5**: You will return to the testing facility for 5 days and the test sites will be evaluated again.

5. RISK & UNFORESEEN RISKS: The test products are intended to come in contact with skin, there might be irritation/allergic or other positive reacti marked for the entire study. Most of these reactions are rapidly reversible. There may be unknown risks, in extreme rare cases, blistering may occur w

7. COMPENSATION FOR INJURY: In the unlikely event that medical treatment is required due to a severe reaction linked to the participation of th treatment will be provided at our discretion when we deem it necessary. Provision of such care is not an admission of legal liability or responsibility for Extended medical care will not be provided.

8. BENEFITS: Participation in this study is **voluntary, you are not an employee of the company and will not be paid at month end.** You will o of the entire study as communicated to you by the **study leader**, the money will be transferred into your account after the first full week FOLLOWING t if the study leader concludes it would be best to discontinue your participation due to a severe reaction, you will be paid in full. If you are dismissed for **you will not be paid. Please note that if your banking details are not complete (including 6 digit branch code) or are not accepted during loa you will receive an sms and the new details will be loaded on the following month's payments.** *Please sign in agreement*

9. CONFIDENTIALITY Reports prepared by Future Cosmetics CC will utilize statistical information only. Confidentiality of any information you pro possible. You are not allowed to any confidential information to any person on the test procedures or products or companies tested.

10. EMERGENCY CONTACT: During the study, should you experience any medical problems, suffer a research-related injury or have questions abo

11. VOLUNTARY PARTICIPATION AND WITHDRAWAL Your participation in this study is entirely voluntary. You may decide not to participat and if you choose to do so, you will be instructed to state your reason. Your participation in this study may be ended without your consent and without reasons: if you fail to follow directions/rules for participating in the study, if it is discovered that you do not meet study requirements. Your participation consent with full or partial compensation (at discretion of the study leader) for the following reasons: If it appears to be medically harmful to you or oth Please confirm that you have read, understood and will comply to the following statement: "Please do not partake in this study if you are HIV positive

12. CONSENT: I have carefully read and understand this informed consent. The test has been explained to my satisfaction and I agree to participa given the opportunity to discuss all aspects of the study and to ask questions. I am at least 18 years old, in good health and freely give my consent to If I have any questions regarding my rights as a research participant I may call the study investigator.

FUTURE COSMETICS CC

From Concept to Product

APPENDIX D: MEDICAL HISTORY FORM (PATCH TESTING) FCSOP100/05 FCSS268

SUBJECT NO:		NAME & SU
SEX: Male Female		RACE:

If any of the following questions is yes, please talk to the study technician at this station.

yes no Do you have any hair/marks/ scars/broken or chapped skin on your face Arm

yes no Are you currently pregnant or breastfeeding?

yes no Do you currently have or do you have a history of psoriasis / eczema?

yes no Do you have any medical conditions, which you are currently under a physician's care for?

Please specify _____

yes no Do you use bath oil, skin c

yes no Have you ever experience disease (reaction to sun)?

yes no Have you had any allergic,

yes no Do you make use of a sun

yes no Have you complied to the

WHAT SKIN PRODUCTS DO YOU USE? Please specify _____

PLEASE INDICATE WHICH MEDICATION HAVE YOU USED WITHIN THE LAST WEEK (W), LAST MONTH (M) or LAST 3 MONTHS (3M)

Allergy Injection	Allergy medication	Antihistamines	Analgesics	Anti-anxiety	
Antidepressants	Anti-diarrhea	Anti-hypertension	Appetite suppressants	Arthritis medication	
Cortisone injections	Cortisone	Asthma medication	Diabetic medicine	Eye drops	
Hormones	Itch relief	Immuno suppressive	Anti-fungal	Muscle ache relief	
Sleep aid products	Pain relievers	Thyroid medication	Tranquillizers	Ulcer medication	

IF ANY ITEMS MARKED, PLEASE LIST SPECIFIC NAMES OR LIST ANY MEDICATION TAKEN WITHIN THE LAST WEEK NOT LISTED ABOVE:

HAVE YOU EVER BEEN TREATED FOR ANY OF THE FOLLOWING:

Eczema	Hair Loss/ Thinning	Keratosis	Acne	Body Fungus	
			Psoriasis	Seborrhea	
PRODUCT SENSITIVITIES:	Deodorants	Sunscreen Products	Eye Cosmetics	Facial Cosmetics	
CANCER:	Skin	OTHER, (please specify)			

If you have marked any of the above, please specify _____

WHAT IS -

YOUR NATURAL COLOUR OF UNTANNED SKIN?	Reddish-white	White Beige/ Milky	Beige/Creamy	
HAIR COLOUR ?	Red, light brown	Blonde, light brown	Brown	
EYE COLOUR?	Light-blue/green/grey	Blue, green, grey	Dark grey, light brown	

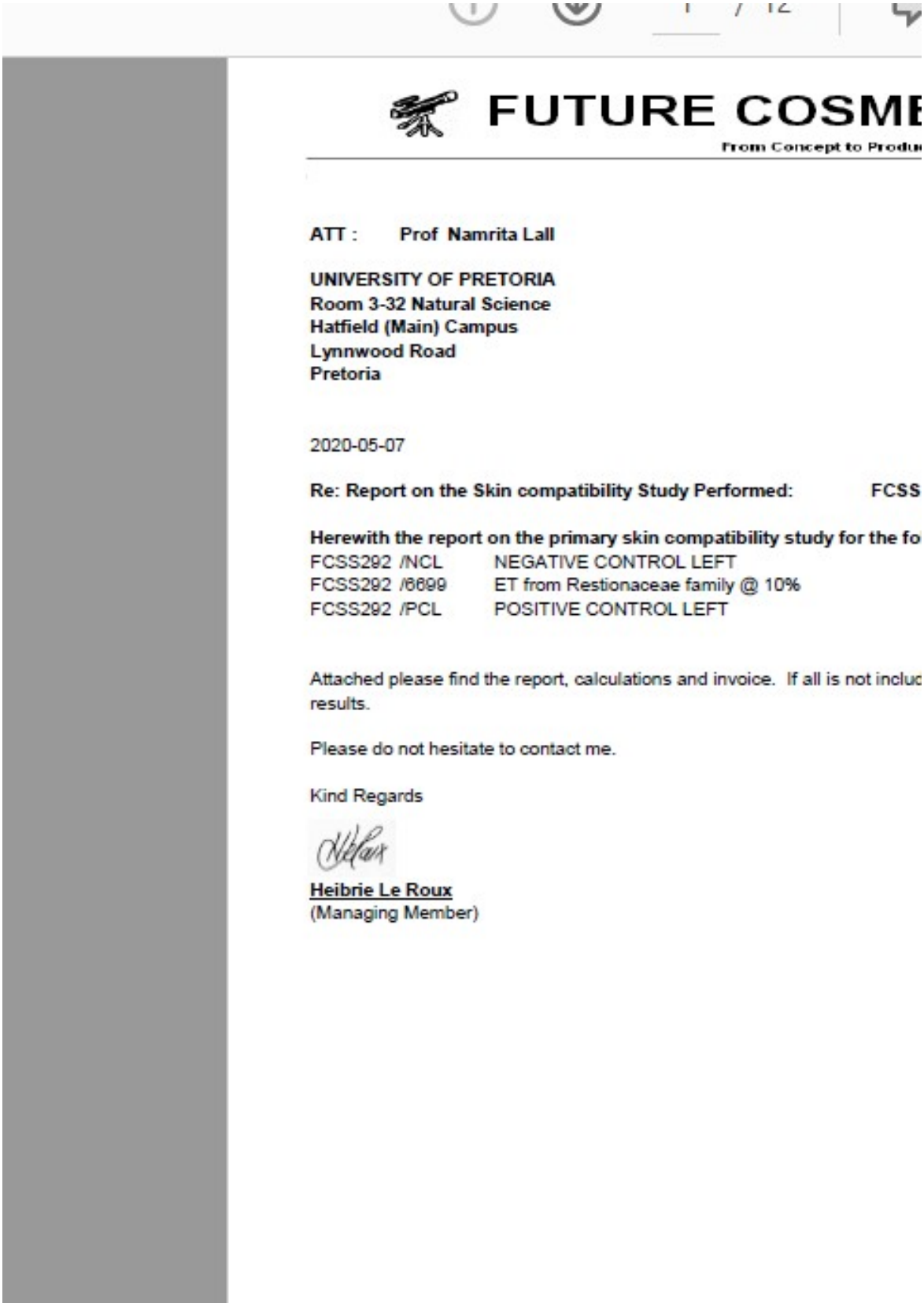
FUTURE COSMETICS

From Concept to Product

INITIATION DATE:	2017/11/2
COMPLETION DATE:	2017/12/0
STUDY NUMBER:	FCSS26

APPENDIX E: DEVIATION F
(REF:FCSOP100/7)

Appendix B2: Irritancy study of *Elegia tectorum* extract in a formulation



FUTURE COSME

From Concept to Produ

ATT : Prof Namrita Lall

UNIVERSITY OF PRETORIA
Room 3-32 Natural Science
Hatfield (Main) Campus
Lynnwood Road
Pretoria

2020-05-07

Re: Report on the Skin compatibility Study Performed: FCSS

Herewith the report on the primary skin compatibility study for the fo

FCSS292 /NCL	NEGATIVE CONTROL LEFT
FCSS292 /6699	ET from Restionaceae family @ 10%
FCSS292 /PCL	POSITIVE CONTROL LEFT

Attached please find the report, calculations and invoice. If all is not incluc
results.

Please do not hesitate to contact me.

Kind Regards

Heibrie Le Roux
(Managing Member)

FUTURE COSME

From Concept to Product

2020-05-07

REPORT ON 24 HOURS OCCLUSIVE COMPATIBILIT

HUMAN PATCH TESTING

STUDY REFERENCE NUMBER: FCSS

Test on primary skin compatibility on human subjects.

Responsible for study:	Heibrie Le Roux Managing Member
Products tested:	FCSS292 /NCL NEGATIVE CONTROL LEF FCSS292 /6699 ET from Restionaceae famil FCSS292 /PCL POSITIVE CONTROL LEFT
Customer:	UNIVERSITY OF PRETORIA Room 3-32 Natural Science Hatfield (Main) Campus Lynnwood Road Pretoria
Concentration of products:	Neat

The following report is an accurate account of the test method as describe
during the course of the study, which was performed within the month pric

The objective of the study was to detect primary skin compatibility potent
subjects. Patch testing represents a relatively safe and reasonable reliab
to a correctly applied patch test could prove that the person experienced

Test Protocol:

Materials:
Test Products
Cotton Buds
Demineralised Water
8mm Finn Chambers
Finn Chamber Templates
Black Marker Pens
Positive Displacement Pipette
Sodium Lauryl Sulphate Solution (1%) as a Positive Control
Demineralised Water as a Negative Control

Camera:

Canon digital camera and Sandisk memory stick.

Products, applicators and sequence:
8mm Finn Chambers on micropore tape was used to allow for occlusive
Where necessary, transpore tape was applied to secure the strips in a m
the test area.
1CC Syringes were used to apply the controls and products.

Method:
Twenty (20) subjects between the ages of 18 and 65 were recruited. (Se
procedure of patch testing was explained to them verbally and each subj
Appendix D : Consent Form and Medical history). Personal details and e
recorded.

Visual assessments of the test sub-sites were made at 24, 48 and 72 hou
inner forearm at 0 hours. The finn chambers covered the patch areas fo
the subject could clean the area by any normal mean.

Colour photographs were taken at each time interval to serve as a record

The controls and product were applied to the inner forearm according to
and recording bias (See Appendix C: Test Substance Sequence).

The following rating system was used to classify the reactions:

0 = No response
0.5 = Minimal/doubtful response

Dermatological Criteria:

Positive reactions of an allergenic nature are profound and clear red, co
severe reactions coalesce into bullae. The diffuse and spread beyond t
usually itching and burning sensations.

Once a reaction has developed, the positive reactions persist for severa
negative at 3 days (72 hours) are often of an irritant nature.

A positive reaction on skin sensitisation has a very similar reaction to th
application (24 hours) if a sensitising species are present, provided abo
been performed therefore appearing at 48 hours; although with some s

The classification of results is interpreted as follows:

Non-irritant = Mean Score (Average plus standard deviation) fa
performed better or similar to that of demineralise

Mild Irritant = Mean Score (Average plus standard deviation) fa
that of positive control. A percentage increase in
given.

Irritant = Mean Score (Average plus standard deviation) fa
performed worse than the positive control.

Tel:+27-128110621 Fax:+27-128110510 Mobile:+27-829613509 E-mail:heibrie@future

FUTURE COSMETICS C

From Concept to Product

Results and Conclusions:

A Summary of the results is given in Attachment E.

TEST RESULTS VALUES AFTER SEVENTY-TWO (72) HOURS

PRODUCT NUMBER	TEST REFERENCE	PRODUCT REFERENCE	TEST PRODUCT NAME:	Average Value
17	FCSS292	/NCL	NEGATIVE CONTROL LEFT	0.28
25	FCSS292	/6699	ET from Restionaceae family @ 10%	0.06
26	FCSS292	/PCL	POSITIVE CONTROL LEFT	1.23

Kind regards

HEIBRIE LE ROUX
(MANAGING MEMBER)

FUTURE COSMETIC

From Concept to Product

Data Sheet of Skin Compatibility Study

INITIATION DATE: 2018/10/21

COMPLETION DATE: 2018/10/24

TEST REF: FCS3292

Table 1: Summary of observations of products and two controls on 20 volunteers

Product 17	FCS3292	/NCL								NEGATIVE CONTROL LEFT				
Volunteer	1	2	3	4	5	6	7	8	9	10	11	12	13	14
24 hours ev 1	0	0	0	0	0	1	0	0	0	0	2.5	2	2	0
24 hours ev 2	0	0	0	0	0	1	0	0	0	0	2.5	2	1	0
48 hours ev 1	0	0	0	0	0	0	0	0.5	0	0	1	0	0	0
48 hours ev 2	0	1	1	0	0	1	0	1	0	0	1	0	0	0
72 hours ev 1	0	0	0	0	0	0.5	0	0.5	0	0	0	0	0.5	0
72 hours ev 2	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0
Average value for product over 72 hours														
Percentage Compatibility Potential														
Number of test subject experiencing reaction after 48 hours														
Subjects	-	2	3	-	-	6	-	8	-	-	11	-	-	-
Range of reactions observed	a mild, spotty or diffused redness												to	
Compatibility Classification	Irritant											Non Irritant		

FUTURE COSMETI

From Concept to Product

Data Sheet of Skin Compatibility Study

INITIATION DATE: 2018/10/21

COMPLETION DATE: 2018/10/24

TEST REF: FCS3292

Table 1: Summary of observations of products and controls on 20 volunteers

Product 25	FCS3292	/6889								ET from Rectionaceae family @ 1				
Volunteer	1	2	3	4	5	6	7	8	9	10	11	12	13	14
24 hours ev 1	0	0	2.5	0	0	0	0	0	0	0	0	0	0	0
24 hours ev 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
48 hours ev 1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
48 hours ev 2	0	0	0	0	0	0	0.5	0	0	0	0	0	0	0
72 hours ev 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72 hours ev 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Average value for product over 72 hours														
Percentage Compatibility Potential														
Number of test subject experiencing reaction after 48 hours														
Subjects	-	-	-	-	-	-	-	-	-	-	-	12	-	-
Range of reactions observed	a mild, spotty or diffused redness												to	
Compatibility Classification	Irritant											Non Irritant		

FUTURE COSMETICS

From Concept to Product

Data Sheet of Skin Compatibility Study

INITIATION DATE: 2018/10/21

COMPLETION DATE: 2018/10/24

TEST REF: FCS3292

Table 1: Summary of observations of products and controls on 20 volunteers

Product 28	FCS3292	/PCL								POSITIVE CONTROL LEFT				
Volunteer	1	2	3	4	5	6	7	8	9	10	11	12	13	14
24 hours ev 1	1	2.5	2.5	0.5	0	2.5	0	0	2.5	1	2.5	3	0.5	0
24 hours ev 2	0	2.5	0	0	0	0	0	0	2.5	0	2.5	3	0	0
48 hours ev 1	1	2.5	2.5	0.5	0	2.5	2	1	2.5	2.5	2.5	2	2.5	2.5
48 hours ev 2	2.5	2.5	1	0	0	0	2.5	1	2.5	2.5	2.5	2.5	0	0
72 hours ev 1	1	0	0	2.5	2.5	2.5	2.5	0	2.5	2.5	2.5	2.5	0	0.5
72 hours ev 2	0.5	0.5	0	0.5	0.5	0	0.5	0	1.5	0	2	2.5	0.5	0

FUTURE COSMET

From Concept to Product

APPE

SUBJECT DEMOGRAPHICS (F

INITIATION DATE:	2019/10/21
COMPLETION DATE:	2019/10/24
STUDY NUMBER:	FCSS292

STUDY AGE RANGE :	18 to 65
TOTAL QUANTITY OF TEST SUBJECTS (n)	20
AVERAGE POPULATION AGE:	46.4
YOUNGEST TEST SUBJECT:	18
OLDEST TEST SUBJECT:	65
QUANTITY OF SUBJECTS PER AGE CATEGORY :	
UNDER 18: n =	0
18 to 20: n =	1
21 to 30: n =	2
31 to 40: n =	2
41 to 50: n =	7
51 to 71: n =	8
STUDY GENDER INFORMATION:	
FEMALE	95.00%
MALE	5.00%
STUDY ETHNICITY INFORMATION	
NEGROID	0%
CAUCASIAN	100%

TEST REF	SUBJECT NUMBER	SKIN TYPE (Fritzpatrick)	AGE
FCSS292	/01	II	52
FCSS292	/02	I	57
FCSS292	/03	II	51
FCSS292	/04	II	56
FCSS292	/05	I	59
FCSS292	/06	II	48
FCSS292	/07	II	39
FCSS292	/08	I	62
FCSS292	/09	II	42
FCSS292	/10	I	48
FCSS292	/11	I	49
FCSS292	/12	II	26
FCSS292	/13	II	18
FCSS292	/14	II	64
FCSS292	/15	II	44

FUTURE COSME

From Concept to Product

AR
TESTING CONDITIONS (F

INITIATION DATE:	2019/10/21
COMPLETION DATE:	2019/10/24
STUDY NUMBER:	FCSS292

DISTRIBUTION OF TESTING CONDIT						
TEMPERATURE (°C)				MEAN		H
22.8				BASELINE		
20.7				T 24HRS		
20.6				T 48HRS		
21.4				T 72HRS		
21.4				TOTAL		
TEMPERATURE RANGE				AMBIENT		H
20.0	to	23.9		RANGE		
20.0				MAXIMUM		
23.9				MINIMUM		
TEMPERATURE (Degrees Celsius)				SUBJECT		
BASELINE	T 24 HRS	T 48 HRS	T 72 HRS	REF:		B
21.8	20.7	20.6	20.0	FCSS292	/01	
22.5	20.6	20.6	20.1	FCSS292	/02	
23.0	20.3	20.5	20.1	FCSS292	/03	
23.9	20.4	20.7	20.2	FCSS292	/04	
23.5	20.3	20.3	20.3	FCSS292	/05	
23.5	20.4	20.0	20.3	FCSS292	/06	
23.3	20.3	20.9	20.7	FCSS292	/07	
23.5	20.4	20.6	20.8	FCSS292	/08	
23.4	20.6	20.0	20.8	FCSS292	/09	
23.0	20.8	20.0	22.1	FCSS292	/10	
23.0	21.0	20.4	22.6	FCSS292	/11	
23.1	21.2	20.6	22.4	FCSS292	/12	
22.3	20.3	20.8	22.5	FCSS292	/13	
22.4	20.6	20.4	22.3	FCSS292	/14	
23.1	20.7	20.3	22.2	FCSS292	/15	
23.5	20.7	20.4	22.2	FCSS292	/16	

FUTURE COSMETICS CC

From Concept to Product

APPENDIX C: TEST SUBSTANCE SEQUENCE (REF:FCSOP100/08)

INITIATION DATE: 2019/10/21
COMPLETION DATE: 2019/10/24
STUDY NUMBER: FCS5292

PRODUCT	PRODUCT NAME:	NO:
/NCL	NEGATIVE CONTROL LEFT	17
/MCL	MARKER CONTROL LEF	22
/6699	ET from Restionaceae family @ 10%	25
/PC	POSITIVE CONTROL LEFT	26

LEFT WRIST	
QO	VO
RO	WO
SO	XO
TO	YO
UO	ZO

TEST SITE:	LEFT VOLAR FOREARM
APPLICATION AMOUNT:	ENOUGH TO FILL FINN CHAMBER
PATCH SIZE:	8mm

SUBJECT NUMBER	LEFT ARM									
	Q	R	S	T	U	V	W	X	Y	Z
/01	17	18	19	20	21	22	23	24	25	26
/02	17	18	19	20	21	22	23	24	25	26
/03	17	18	19	20	21	22	23	24	25	26
/04	26	17	18	19	20	21	22	23	24	25
/05	26	17	18	19	20	21	22	23	24	25
/06	26	17	18	19	20	21	22	23	24	25
/07	25	26	17	18	19	20	21	22	23	24
/08	25	26	17	18	19	20	21	22	23	24

FUTURE COSMETICS CC

From Concept to Product

APPENDIX D: CONSENT FORM (PATCH TESTING) FCSOP100/05	FCSS292	SUBJECT NO:	NAME

1. INTRODUCTION: Before agreeing to participate in this study, it is important that you read & understand the following explanation and procedures. Yo guarantees or assurances can be given to the results of this study.

2. BACKGROUND: You have been invited to participate in this study designed to evaluate the performance of one/ more products to be tested for irrita in good health and willing to follow study guidelines/rules (**including medication restrictions & avoidance of water) as well as not covering the test** You are not allowed to apply any products, exercise or wet the test sites. In addition if you have any reactions, allergies to cosmetics/ preservatives/frag interfere with the study or use any medication that might affect results or if you are pregnant, you cannot participate in this study. The minimum amount

3. RULES TO COMPLY WITH:

You will be asked to abandon the study without compensation if you have wiped or washed your arm during the first 24 hours. *Please sign if a*
I am not currently busy with any other study or will not apply for any others study during this study duration. *Please sign if agree*
I agree to refrain from using any topical products on my arm, or to wash my arm for the first 24 hours. *Please sign if agree*

4. DURATION OF EVALUATION AND PROCEDURES: **Day 1**: You will be asked to read and sign the consent form and your medical history will b study. If you meet the inclusion criteria, you must sign an attendance register. The test sites will be examined for hair,bruises,scarring etc. If you qualify a series of products will be applied. **Day 2, 3, 4 and 5**: You will return to the testing facility for 5 days and the test sites will be evaluated again.

5. RISK & UNFORESEEN RISKS: The test products are intended to come in contact with skin, there might be irritation/allergic or other positive reactio marked for the entire study. Most of these reactions are rapidly reversible. There may be unknown risks, in extreme rare cases, blistering may occur wh

7. COMPENSATION FOR INJURY; In the unlikely event that medical treatment is required due to a severe reaction linked to the participation of this treatment will be provided at our discretion when we deem it necessary. Provision of such care is not an admission of legal liability or responsibility for th Extended medical care will not be provided.

8. BENEFITS; Participation in this study is **voluntary, you are not an employee of the company and will not be paid at month end.** You will onl of the entire study as communicated to you by the **study leader**, the money will be transferred into your account after the first full week FOLLOWING th If the study leader concludes it would be best to discontinue your participation due to a severe reaction, you will be paid in full. If you are dismissed for n **you will not be paid. Please note that if your banking details are not complete (including 6 digit branch code) or are not accepted during load you will receive an sms and the new details will be loaded on the following month's payments.** *Please sign in agreement to*

9. CONFIDENTIALITY Reports prepared by Future Cosmetics CC will utilize statistical information only. Confidentiality of any information you provid possible. You are not allowed to any confidential information to any person on the test procedures or products or companies tested.

10. EMERGENCY CONTACT; During the study, should you experience any medical problems, suffer a research-related injury or have questions about

11. VOLUNTARY PARTICIPATION AND WITHDRAWAL Your participation in this study is entirely voluntary. You may decide not to participate o and if you choose to do so, you will be instructed to state your reason. Your participation in this study may be ended without your consent and without co reasons: if you fail to follow directions/rules for participating in the study, if it is discovered that you do not meet study requirements. Your participation in consent with full or partial compensation (at discretion of the study leader) for the following reasons: if it appears to be medically harmful to you or other Please confirm that you have read, understood and will comply to the following statement: "Please do not partake in this study if you are HIV positive or

12. CONSENT: I have carefully read and understand this informed consent. The test has been explained to my satisfaction and I agree to participate given the opportunity to discuss all aspects of the study and to ask questions. I am at least 18 years old, in good health and freely give my consent to se

FUTURE COSMETICS CC

From Concept to Product

APPENDIX D: MEDICAL HISTORY FORM (PATCH TESTING) FCSOP100/05 FCSS292

SUBJECT NO:		NAME & SURN
SEX: Male	Female	RACE:

If any of the following questions is yes, please talk to the study technician at this station.

yes no Do you have any hair/marks/ scars/broken or chapped skin on your face Arm

yes no Are you currently pregnant or breastfeeding?

yes no Do you currently have or do you have a history of psoriasis / eczema?

yes no Do you have any medical conditions, which you are currently under a physician's care for?

Please specify _____

yes no Do you use bath oil, skin care

yes no Have you ever experienced a disease (reaction to sun)?

yes no Have you had any allergic, se

yes no Do you make use of a sun be

yes no Have you complied to the 3 d

WHAT SKIN PRODUCTS DO YOU USE? Please specify _____

PLEASE INDICATE WHICH MEDICATION HAVE YOU USED WITHIN THE LAST WEEK (W), LAST MONTH (M) or LAST 3 MONTHS (3M)

Allergy Injection	Allergy medication	Antihistamines	Analgesics	Anti-anxiety	Anti
Antidepressants	Anti-diarrhea	Anti-hypertension	Appetite suppressants	Arthritis medication	Hea
Cortisone injections	Cortisone	Asthma medication	Diabetic medicine	Eye drops	Gou
Hormones	Itch relief	Immuno suppressive	Anti-fungal	Muscle ache relief	Pro
Sleep aid products	Pain relievers	Thyroid medication	Tranquillizers	Ulcer medication	

IF ANY ITEMS MARKED, PLEASE LIST SPECIFIC NAMES OR LIST ANY MEDICATION TAKEN WITHIN THE LAST WEEK NOT LISTED ABOVE: _____

HAVE YOU EVER BEEN TREATED FOR ANY OF THE FOLLOWING:

Eczema	Hair Loss/ Thinning	Keratosis	Acne	Body Fungus	Chr
			Psoriasis	Seborrhea	Skir

PRODUCT SENSITIVITIES:	Deodorants	Sunscreen Products	Eye Cosmetics	Facial Cosmetics	Frag
CANCER:	Skin	OTHER, (please specify) _____			

If you have marked any of the above, please specify _____

WHAT IS - YOUR NATURAL COLOUR OF UNTANNED SKIN?	Reddish-white	White Beige/ Milky	Beige/Creamy	Ligh
HAIR COLOUR ?	Red, light brown	Blonde, light brown	Brown	Dar
EYE COLOUR?	Light-blue/green/grey	Blue, green, grey	Dark grey, light brown	Bro

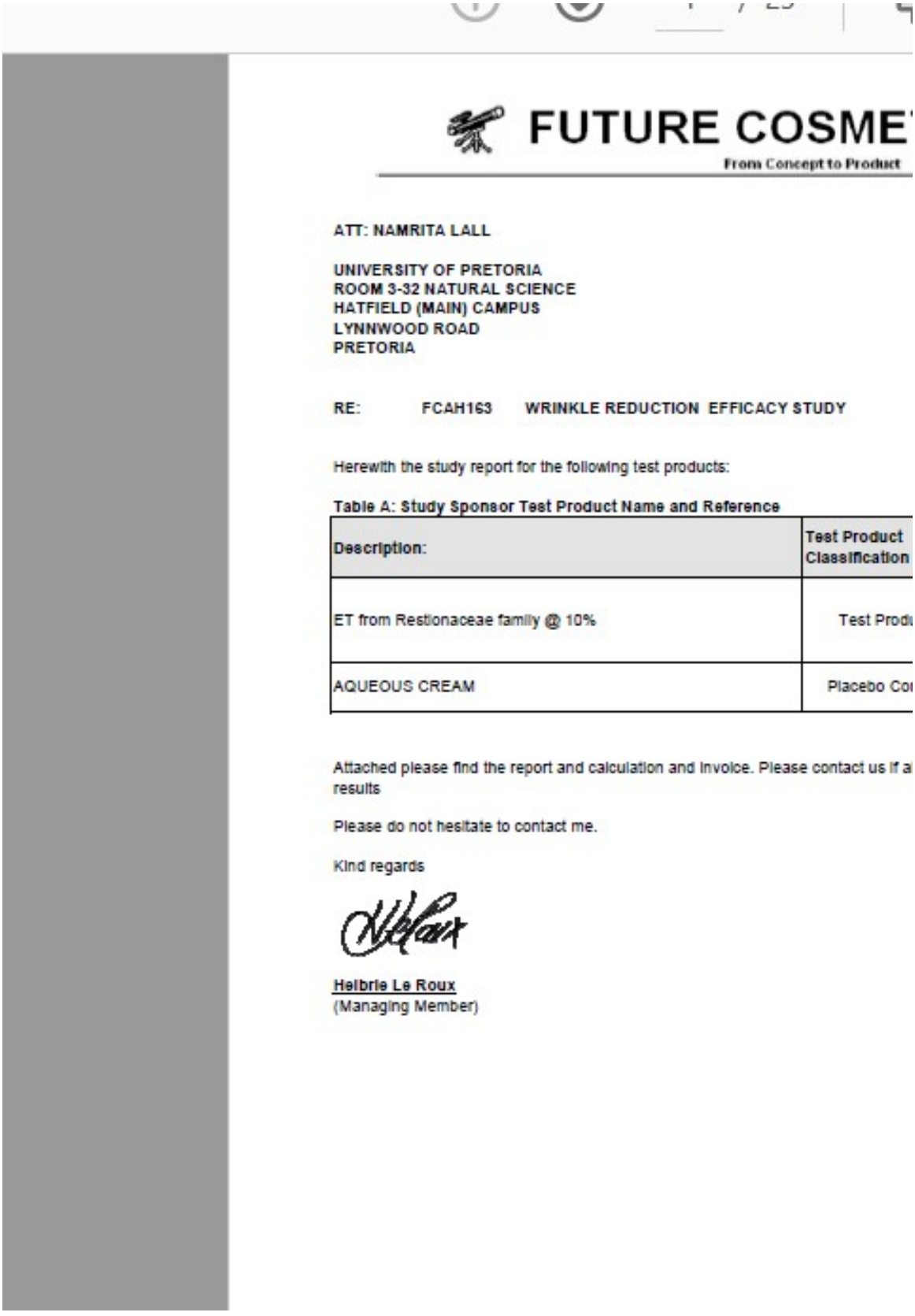
FUTURE COSMETIC

From Concept to Product

INITIATION DATE: _____
COMPLETION DATE: _____
STUDY NUMBER: _____

APPENDIX E: DEVIATI
(REF:FCSOP10

Appendix C: Efficacy studies in *in vivo*, of the *Elegia tectorum*



FUTURE COSME
From Concept to Product

ATT: NAMRITA LALL

UNIVERSITY OF PRETORIA
ROOM 3-32 NATURAL SCIENCE
HATFIELD (MAIN) CAMPUS
LYNNWOOD ROAD
PRETORIA

RE: FCAH163 WRINKLE REDUCTION EFFICACY STUDY

Herewith the study report for the following test products:

Table A: Study Sponsor Test Product Name and Reference

Description:	Test Product Classification
ET from Restionaceae family @ 10%	Test Prod
AQUEOUS CREAM	Placebo Co

Attached please find the report and calculation and invoice. Please contact us if a
results

Please do not hesitate to contact me.

Kind regards

Helbrie Le Roux
(Managing Member)

FUTURE COSME

From Concept to Product

TABLE OF CONTENTS:

FUTURE COSME

From Concept to Product

SUMMARY OVERVIEW

The experimental plan was to evaluate the test product's effi

by conducting an objective, double-blinded in viv

The demographics of the homogenous test poluiation was

I - III

The VISIOSCAN VC98 device was used fo

Photographs serve as a recording of the event. Evaluations were done at

FUTURE COSME

From Concept to Product

STUDY INFORMATION	
Title of the study:	WRINKLE REDUCTION EFFICACY STUDY
Name of the Investigator & Address:	FUTURE COSMETICS CC 287 Sinovich Street Grootfontein Country Estate Garsfontein Road Off Garsfontein Road Pretoria 0081
Sponsor Sponsor address:	ATT: NAMRITA LALL UNIVERSITY OF PRETORIA ROOM 3-32 NATURAL SCIENCE HATFIELD (MAIN) CAMPUS LYNNWOOD ROAD PRETORIA
Test and Product Nr:	FCAH
Test Product Name	ET from Restiona
Testing required	WRINKLE REDUCTI
Application Amount	0
Placebo Reference	FCAH
Testing objective:	The objective of this study was to determine the test pro - WRINKLE REDUCTION
Testing initation date	201
Testing end date	201
OBTAINED ACTUAL RESULTS	FCAH163/6699
	WRINKLE REDUCTION

FUTURE COSME

From Concept to Product

AUTHENTICATION:

I, the undersigned, hereby declare that the work performed in this st
procedures described herein and this report represents a true accur

Approval	Signat
Responsible for the Study in South Africa **HEIBRIE LE ROUX** (Principal Investigator)	
Quality Assurance Officer: HEIBRIE LE ROUX	
Study Coordinator: HEIBRIE LE ROUX	
Quality Control: HEIBRIE LE ROUX	
Technician: FUNANANI NETHAMBA	
Technician: MELITA MANAMELA	

FUTURE COSME

Table B: Management Summary		
Test Product vs. Control Evaluation	Time Interval	Para
ET from Restionaceae family @ 10% FCAH163/6699 **compared to** FCAH163/6705 AQUEOUS CREAM	DAY 14:	W
	DAY 28:	W

Attached please find the report and calculations and invoice. Please contact us if all is not include

Please do not hesitate to contact me.

Kind Regards

Helbrie Le Roux
Managing Member

FUTURE COSME

From Concept to Product

2020-05-07

REPORT ON

WRINKLE REDUCTION EFFICACY

REF: FCAH163

Responsible for Study: HEIBRIE LE ROUX
Managing Member

Study Technicians: FUNANANI NETHAM
MELITA MANAMELA

Quality Control and Report Writing: JOSUA LE ROUX

Quality Assurance: HEIBRIE LE ROUX

Testing Facility: Future Cosmetics CC
267 Sinovich Street
Grootfontein Country
Garsfontein Road
Off Garsfontein Road
Pretoria
0081

Test Products:

FCAH163/6699	ET
FCAH163/6705	AQ

Concentration of Test Product: Neat

FUTURE COSME

From Concept to Product

Objective:
The objective of the study was to determine the product's efficacy to
- WRINKLE REDUCTION

The evaluations were performed in a double-blind manner via instrumental techniques at baseline
after different time interval periods as prescribed by study sponsor.

Test Protocol:
This report is an accurate account of the test method used, (as described by the test protocol) an
The study was performed within the months prior to the date of this report. The protocol is availab
Appendix H: Deviation

Materials:
Test Products as per Table A (page1 of the report)
Colour Coded Syringes

Instrumentation:
VISIOSCAN VC98 (SN14315051)
HISENSE AIR CONDITIONING UNIT Model: AS-12UR4SVNVG2
ECOAIRE AIR CONDITIONING UNIT Model: EC12/CC12
OLYMPIA SPLENDID AQUADRY 28 DEHUMIDIFIER
VISIOFACE RD (SN17486645)

Testing Conditions:
The study was carried out under controlled conditions with the following mean values:

Temperature Range: (°C)	20.00	to	24.7
Humidity Range (%RH)	32.10	to	51.9

(Please see Appendix B: Testing Conditions)

Test Subject Demographics:
A group of test subjects between the ages of 29 - 64 were recruited and accepted into the test pa
as specified by the in-house protocol.

The quantity of valid test subjects that completed the study was

The subjects complied with the rules and specifications of the study and all results recorded were
Subject Demographics).

Please see Appendix A: Subject Demographics for test subject drop-out or elimination schedule

Method:

The procedure of testing was explained to them verbally and a form of consent and medical hist
subject's assessment of their own skin type were recorded. (See Appendix C: The Consent For

Colour photographs were taken at each time interval to serve as a recording of the study condu

All Subjects were instructed to rest for twenty (20) minutes before any testing was performed.
The designated left and right test sub-sites were cleansed with alcohol and allowed to air dry for

The designated left and right test sub-sites were demarcated with surgical marker as per test su
Sequence)

The temperature and relative humidity were recorded during the time of the study and noted do

At least three (3) measurements were taken at each test sub-site at each time interval and save
further calculations and statistical evaluation.

The instrumental visioscan VC98 was gently sterilised with a tissue and alcohol, between all tes

A study technician trained the test subjects in applying the test products evenly to the test sites,
Substance Sequence).

Test subjects were instructed to apply the test substances twice a day as per application instruc

Subjects were restricted from using any topical products or medication not approved by the stud

Subjects were informed when to return to the testing facility for each time interval evaluation.

Visioscan photos for designated time intervals were taken at the different designated test sites.

FUTURE COSME

From Concept to Product

Research Design:

VISIOSCAN

The Visioscan calculates from an eight (8) bit greyscale captured image, which defines intensity smoothness, scaliness, wrinkles etc. The change in the parameter of captured images is calculat reduction intensity. Readings will be taken on each designated spot in each test site to serve as study.

Data Sampling:

A sample can be defined as a subset of the whole population which is investigated by a research population (Bless & Higson-Smith, 1995). A sampling plan can be described as a design, schem are to be selected in a study (Rosnow & Rosenthal, 1996). A distinction is made between probat made of a non-probability sampling method. A non-probability sampling method can be describec sample based in some part on the judgment of the researcher (Kinnear & Taylor, 1996). The rese age. A sample of convenience was used in this study.

Statistical Data Analysis Procedure:

Data Analysis:

The data was captured onto excel and converted to extended excel statistical tests in order to do

1 - To first determine whether the data was distributed normally.
2 - To determine whether significant differences existed between the test product ar

Since the sample was relatively small, use was made of a parametric test, the (unequal or equal test is used to determine whether a given treatment had a significant effect on a population. (Keil data the Wilcoxon Signed Rank Sum Test will be used.

The following statistical data analysis procedures were used:

Descriptive Analysis: Descriptive statistics are primarily aimed at describing the data

Inferential statistics: Test hypotheses about differences in two (2) populations on the human skin subjects.
(Tabachnick & Fidell, 1996: p.9).

Statistically significant differences between variables are indicated by a significance value p. If th indication that there is a statistically significant difference, at the 5% level of confidence.

Results:

Descriptive Analysis:
Descriptive Analysis entails the ordering and summarizing of data by means of tabulation and th

A Summary of the individual results recorded during the study for all test products, as well as all

INSTRUMENTAL EVALUATION **VISIOSCAN VC98 (SN14315051)**

Table F1 DESCRIPTIVE ANALYSIS OF VISIOSCAN VC98 (WRINKLE REDUCTION) VAL

Table F2 DESCRIPTIVE ANALYSIS OF VISIOSCAN VC98 (WRINKLE REDUCTION) VAL

Inferential Statistics:
Statistical Inference draws conclusions about the population from which the sample was drawn b

A Summary of the Statistical Analysis is given in Appendix G:

INSTRUMENTAL EVALUATION **VISIOSCAN VC98 (SN14315051)**

Table G1 STATISTICAL ANALYSIS OF VISIOSCAN VC98 (WRINKLE REDUCTION) FOR FCAH163/6699 vs PLACEBO CONTROL FCAH163/6705 for BASELINE

Table G2 STATISTICAL ANALYSIS OF VISIOSCAN VC98 (WRINKLE REDUCTION) FOR FCAH163/6699 vs PLACEBO CONTROL FCAH163/6705 for DAY 14

Table G3 STATISTICAL ANALYSIS OF VISIOSCAN VC98 (WRINKLE REDUCTION) FOR FCAH163/6699 vs PLACEBO CONTROL FCAH163/6705 for DAY 28

Table B: Comparison of VISIOSCAN VC98 values for the test

WRINKLE REDUCTION

Mean (± Standard Deviation)

Time Interval	FCAH163/6699 TEST PRODUCT	Table Ref.	FC
BASELINE (BL)	760.82	Table F1	
STANDARD DEVIATION	433.92		
DIFFERENCE (DAY 14 - BL)	-117.75	Table F1	
STANDARD DEVIATION	381.51		
DIFFERENCE (DAY 28 - BL)	-161.63	Table F1	
STANDARD DEVIATION	419.83		

Conclusions: WRINKLE REDUCTION

BASELINE:
A Wilcoxon Signed Rank Sum Test was performed to compare the treated and untreated control
a statistical significant difference on a 5% level of confidence. The test product sites were signif
consequence on baseline as analysis done on absolute value

DAY 14:
A t-Test was performed to determine the treatment effect by comparing the average difference v
and there was
a statistical significant difference on a 5% level of confidence. The test product sites were signif

DAY 28:
A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by compari
treated and control test-sites, and there was
a statistical significant difference on a 5% level of confidence. The test product sites were signif

It can therefore be concluded that the test product was effective in decreasing the wrinkle consecutive use (twice a day) when compared to a placebo control.

FUTURE COSME

From Concept to Product

INITIATION DATE:	2019/11/01
COMPLETION DATE:	2019/11/29
STUDY NUMBER:	FCAH163

APPENDIX A: SUBJECT DEI

DISTRIBUTION OF TEST SUBJECT DEMOGRAPHICS WHICH WAS INCLUDED INTO THE CALCULATIONS:		TEST SUBJE CALCULATI
TOTAL: n =	26	TEST SUBJ RE
AVERAGE AGE:	50.0	SUB 04
MIN AGE:	29	
MAX AGE:	64	
STUDY AGE RANGE :	28 to 64	
FEMALE	100%	
MALE	0	
ETHNIC BLACK	0%	
CAUCASIAN	100%	
UNDER 18: n =	0	
18 to 20: n =	0	
21 to 30: n =	1	
31 to 40: n =	2	
41 to 50: n =	11	
51 to 71: n =	11	

TEST REFERENCE	SUBJECT NUMBER	SKIN TYPE (Fritzpatrick)	AGE
FCAH163	/01	II	57
FCAH163	/02	II	51
FCAH163	/03	II	49
FCAH163	/05	II	57
FCAH163	/06	III	52
FCAH163	/07	II	51
FCAH163	/08	I	53
FCAH163	/09	II	47
FCAH163	/10	II	64
FCAH163	/11	II	62
FCAH163	/12	II	43
FCAH163	/13	II	64
FCAH163	/14	II	47
FCAH163	/15	III	62

FUTURE COSME

From Concept to Product

INITIATION DATE: 201
COMPLETION DATE: 201
STUDY NUMBER: FCA

APPENDIX B: TESTING C

DISTRIBUTION OF TESTING CONDITIONS:

DISTRIBUTION OF TESTING CONDITIONS:	TEMPERATURE Degrees Celsius (20-22C) (Mean)		
TIME INTERVAL	BASELINE:	DAY 14:	DAY 28:
MEAN	21.12	21.79	23.76
TOTAL MEAN	22.27		
MIN VALUE	20.20		
MAX VALUE	24.70		
TEMPERATURE RANGE	20.2 TO 24.7		

TEST REFERENCE	SUBJECT NUMBER	TEMPERATURE (Degrees Celsius 20-22C)		
		BASELINE:	DAY 14:	DAY 28:
FCAH163	/01	21.4	21.0	23.1
FCAH163	/02	21.4	21.0	23.1
FCAH163	/03	21.0		23.1
FCAH163	/05			23.2
FCAH163	/06			23.2
FCAH163	/07			23.4
FCAH163	/08	21.7		23.4
FCAH163	/09	21.7		23.9
FCAH163	/10	20.4	21.6	23.5
FCAH163	/11		21.7	23.5
FCAH163	/12	20.4	21.9	23.6
FCAH163	/13	20.5	21.9	23.6
FCAH163	/14	20.4	21.9	23.5
FCAH163	/15	20.4	21.9	24.0
FCAH163	/16	20.6	22.0	24.3
FCAH163	/17	20.2	21.9	24.4
FCAH163	/18	21.9	22.4	
FCAH163	/19			

FUTURE COSMETICS CC

From Concept to Product

APPENDIX C: INFORMED CONSENT FORM: WRINKLE REDUCTION

PROTOCOL NO:	FCAH163	SUBJECT NO:	FULL NAME & SURNAME:	ID/PAS
		To be assigned by admission staff		NATIO

1. INTRODUCTION: Before agreeing to participate in this study, it is important that you read & understand the following explanation and procedures. You have the right to withdraw from this study at any tim results of this study.

2. BACKGROUND: You have been invited to participate in this study designed to evaluate the performance of one/more products to be tested for its efficacy to improve wrinkle reduction.. You must be at least study guidelines/rules (including medication restrictions). In addition if you have any reactions, allergies to cosmetics/ preservatives/fragrance or have any condition that might interfere with the study or use pregnant/breastfeeding, you cannot participate in this study.
The minimum amount of volunteers required for this study is 24

3. RULES TO COMPLY WITH:
PLEASE READ THE FOLLOWING STATEMENTS CAREFULLY AND SIGN:

	Please reply to each Block Yes/No/OK	Volu
I have complied with the "non-smoking an hour before the test" rule. Please sign if agree		
I am not currently busy with any other study or have not been doing a similar study in the last four (4) weeks. Please sign if agree		
I am not on cortisone medication. Please sign if agree		
I agree to refrain from using any other creams on test area in the morning AND evening except for the cream supplied. Please sign if agree		
I do not have/or are aware of any skin disease. Please sign if agree		
I am not being treated for asthma. Please sign if agree		
I am not receiving systemic or topical drugs or medication , which can influence the study results. Please sign if agree		
I have used moisturizer. Please sign if agree		
I have used foundation. Please sign if agree		
I have used sunscreens. Please sign if agree		

4. DURATION OF EVALUATION AND PROCEDURES: Baseline (DAY 0) : You will be asked to read and sign the informed consent form (Appendix C) and your medical history (Appendix D) will be reviewed to inclusion criteria, you must sign an attendance register. The test sites will be examined for hair,bruises,scarring etc. If you qualify a battery of tests will be performed, with test product application at the test facility which time intervals you need to return to the testing facility for further evaluations. The approximate time for visit is 1-2 hours.DAY 14: You will asked to sign attendance register. Relevant test procedures to hours. DAY 28: You will asked to sign attendance register. Relevant test procedures to be performed. Approximate time for procedures is 2 hours.

5. RISK & UNFORESEEN RISKS: The test products are intended to come in contact with skin, there might be irritation/allergic or other positive reactions to the test sites, the test sites will be marked for reversible. There may be unknown risks, in extreme rare cases, blistering may occur which might cause blemishes/ scarring.

6. COMPENSATION FOR INJURY: In the unlikely event that medical treatment is required due to a severe reaction linked to the participation of this test, appropriate and reasonable medical treatment will be pro Provision of such care is not an admission of legal liability or responsibility for the condition being treated. Extended medical care will not be provided.

7. BENEFITS: Participation in this study is voluntary, you are not an employee of the company and will not be compensated at month end. You will only be paid after the completion of the entire study as comm transferred into your account after the testing has been completed. If the study leader concludes it would be best to discontinue your participation due to a severe reaction, you will be paid in full. If you are dism you will not be paid.

8. CONFIDENTIALITY: Reports prepared by Future Cosmetics CC will utilize descriptive information only. Confidentiality of any information you provide will be maintained to the maximum extent possible. You to any person on the test procedures or products or companies tested.

FUTURE COSMETICS CC

From Concept to Product

APPENDIX D:

MEDICAL HISTORY FORM for PROTOCOL NO: FCAH163	SUBJECT NO:		NAME & SURNAME:	
	SEX: Male	Female	RACE:	Caucasian

If any of the following questions is yes, please talk to the study technician at this station.

yes	no	Do you have any hair/ marks/ scars/broken or chapped skin on your face	3.22	yes	no	Do you use any bath oils, skincare lotion or other cosmetic
yes	no	Are you currently pregnant or breastfeeding?	3.24	yes	no	Have you ever experienced a phototoxic/photo allergenic r (reaction to sun)?
yes	no	Do you currently have or do you have a history of psoriasis / eczema?	3.26	yes	no	Have you had any allergic, sensitive reactions to skin prod
yes	no	Do you have any medical conditions, which you are currently under a physician's care for?	3.28	yes	no	Do you make use of a sunbed? If yes, when was your last

Please specify if YES to any of above was mentioned? _____

Please specify if YES to any of above was mentioned? _____

WHAT SKIN CARE PRODUCTS DO YOU USE? *Please specify* _____

WHAT SOAPS/PRODUCTS HAVE YOU USED IN THE LAST FOUR (4) WEEKS? *Please specify* _____

PLEASE INDICATE WHICH MEDICATION HAVE YOU USED WITHIN THE LAST WEEK (W), LAST MONTH (M) or LAST 3 MONTHS (3M)

☐ Allergy Injection	☐ Allergy medication	☐ Antihistamines	☐ Analgesics	☐ Anti-anxiety	☐ Anti-seizure	☐ Antibi
☐ Antidepressants	☐ Anti-diarrhea	☐ Anti-hypertension	☐ Appetite suppressants	☐ Arthritis medication	☐ Anti-fungal	☐ Blood
☐ Cortisone injections	☐ Cortisone	☐ Diuretics	☐ Diabetic medicine	☐ Eye drops	☐ Gout medication	☐ Heada
☐ Hormones	☐ Itch relief	☐ Immuno suppressive	☐ Laxative	☐ Muscle ache relief	☐ Prostate medication	☐ Prem
☐ Sleep aid products	☐ Steroids	☐ Thyroid medication	☐ Tranquillizers	☐ Ulcer medication	☐ Contraceptive	

IF ANY ITEMS MARKED, PLEASE LIST SPECIFIC NAMES OR LIST ANY MEDICATION TAKEN WITHIN THE LAST WEEK NOT LISTED ABOVE: _____

SKIN CONDITION:	☐ Oily (cheeks, forehead, chin)	☐ Normal/Combination (oily T panel)	☐ Dry, Scaly	☐ Sensitive	☐ Known to have positive react

HAVE YOU EVER BEEN TREATED FOR ANY OF THE FOLLOWING:

☐ Eczema	☐ Hair Loss/ Thinnig	☐ Keratosis	☐ Acne	☐ Body Fungus	☐ Chronic Dry Skin	☐ Cold S
			☐ Psoriasis	☐ Seborrhea	☐ Skin irritation	☐ Sensit

PRODUCT SENSITIVITIES: ☐ Deodorants ☐ Soaps ☐ Eye Cosmetics ☐ Facial Cosmetics ☐ Fragrances ☐ Moist

CANCER: ☐ Skin ☐ OTHER, (please specify) _____

If you have marked any of the above, please specify _____

	Score1	Score 1.5	Score 2	Score 3	Score 4

FUTURE COSMET

From Concept to Product

INITIATION DATE:	2019/11/01
COMPLETION DATE:	2019/11/29
STUDY NUMBER:	FCAH163

TEST SUBSTANCE SEQUENCE

TEST REFERENCE:	PRODUCT REFERENCE:	PRODUCT NAME:
FCAH163	/6699	ET from Restionaceae family @ 10%
FCAH163	/6705	AQUEOUS CREAM

APPLICATION AMOUNT:	0.6 grams
TEST SUB SITE:	HALF FACE

TEST REFERENCE	SUBJECT NUMBER	LEFT HALF FACE	RIGHT HALF FACE
FCAH163	/01	2	1
FCAH163	/02	1	2
FCAH163	/03	2	1
FCAH163	/05	1	2
FCAH163	/06	1	2
FCAH163	/07	2	1
FCAH163	/08	1	2
FCAH163	/09	2	1
FCAH163	/10	1	2
FCAH163	/11	2	1
FCAH163	/12	1	2
FCAH163	/13	1	2
FCAH163	/14	1	2
FCAH163	/15	2	1
FCAH163	/16	1	2
FCAH163	/17	1	2

FUTURE COSME

From Concept to Product

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FUTURE COSMETI

From Concept to Product

INITIATION DATE:	2019/11/01
COMPLETION DATE:	2019/11/29
TEST SITE:	HALF FACE
PARAMETER:	WRINKLE REDUCTION
EVALUATION TYPE:	VISIOSCAN VC98 (SN14315051)
STUDY NUMBER:	FCAH163

Table F1 DESCRIPTIVE ANALYSIS OF VISIOSCAN VC98 (WRINKLE REDUCTION) VALUES FOR

	AVERAGE
AVERAGE	760.82
STANDARD DEVIATION	433.92
QTY OF DATA POINTS	110
QTY OF TEST SUBJECTS	25

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	Baseline		AVG BL	Day 14		AVG
			Reading-1	Reading-2		Reading-1	Reading-2	
1	1	RW1	990	519	**454.50**	1403	594	99
1	1	RW2	678	623	**650.50**	780	1628	119
2	1	LW1	2690	776	**1733.00**	818	799	80
2	1	LW2	1768	953	**1360.50**	1406	257	83
3	1	RW1	622	937	**779.50**	719	534	62
3	1	RW2	512	481	**496.50**	1119	592	85
5	1	LW1	582	1341	**961.50**	709	318	51
5	1	LW2	429	525	**477.00**	986	429	39
5	1	LW3	1113	937	**1025.00**	343	114	22
6	1	LW1	760	525	**642.50**	579	448	51
6	1	LW2	2991	1278	**2134.50**	1483	647	106
6	1	LW3	2845	1493	**2169.00**	2987	1087	202
7	1	RW1	378	706	**542.00**	991	401	39
7	1	RW2	283	251	**267.00**	603	422	51
7	1	RW3	508	513	**510.50**	885	370	62
8	1	LW1	487	644	**565.50**	519	644	58
8	1	LW2	1005	561	**783.00**	568	158	36
8	1	LW3	597	429		599	557	
9	1	RW1	561	440	**500.50**	933	1162	104
9	1	RW2	438	238	**338.00**	976	743	85
9	1	RW3	810	294	**552.00**	795	700	74
10	1	LW1	479	623	**551.00**	372	657	51
10	1	LW2	760	624	**692.00**	344	548	44
10	1	LW3	256	612	**434.00**	484	434	44
11	1	RW1	573	461	**517.00**	381	533	45
11	1	RW2	1726	790	**1258.00**	491	956	72
12	1	LW1	512	342	**427.00**	394	411	40
12	1	LW2	1014	279	**646.50**	423	373	39
13	1	LW1	739	443	**591.00**	821	1032	92
13	1	LW2	871	888	**879.50**	403	269	33
14	1	LW1	149	371	**260.00**	683	443	55
15	1	RW1	498	457	**477.50**	819	285	55
15	1	RW2	597	461	**529.00**	215	175	19
16	1	LW1	1573	825	**1199.00**	440	809	62
16	1	LW2	1096	594	**845.00**	1207	803	100
17	1	LW1	1285	1051	**1158.00**	957	999	97
17	1	LW2	673	272	**472.50**	850	547	69
18	1	LW1	821	1028	**924.50**	531	330	43
18	1	LW2	272	259	**265.50**	401	415	40
19	1	RW1	645	826	**735.50**	673	389	53
19	1	RW2	484	273	**378.50**	1435	530	98
20	1	LW1	1092	1278	**1185.00**	472	661	56
20	1	LW2	2106	744	**1425.00**	1493	1145	131

FUTURE COSMETI

From Concept to Product

INITIATION DATE:	2019/11/01
COMPLETION DATE:	2019/11/29
TEST SITE:	HALF FACE
PARAMETER:	WRINKLE REDUCTION
EVALUATION TYPE:	VISIOSCAN VC98 (SN14315051)
STUDY NUMBER:	FCAH163

Table F2 DESCRIPTIVE ANALYSIS OF VISIOSCAN VC98 (WRINKLE REDUCTION) VALUES FOR

	AVERAGE
AVERAGE	632.47
STANDARD DEVIATION	276.96
QTY OF DATA POINTS	112
QTY OF TEST SUBJECTS	25

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	Baseline		AVG BL	Day 14		Av
			Reading-1	Reading-2		Reading-1	Reading-2	
1	2	LW1	690	467	578.50	828	348	5
1	2	LW2	1291	483	887.00	820	454	6
2	2	RW1	662	519	590.50	985	433	6
2	2	RW2	1741	1113	1427.00	696	956	8
3	2	LW1	1130	1235	1182.50	1601	984	12
3	2	LW2	986	668	827.00	1511	799	11
5	2	RW1	680	315	497.50	670	548	6
5	2	RW2	1858	957	1407.50	813	632	7
5	2	RW3	1350	933	1141.50	803	614	7
6	2	RW1	774	334	554.00	1085	912	9
6	2	RW2	728	645	686.50	384	378	3
6	2	RW3	622	335	478.50	1080	652	8
7	2	LW1	833	780	806.50	1008	370	6
7	2	LW2	641	528	584.50	756	522	6
7	2	LW3	1266	783	1024.50	428	483	4
8	2	RW1	502	589	545.50	901	744	8
8	2	RW2	474	352	413.00	328	183	2
9	2	LW1	457	319	388.00	1045	852	9
9	2	LW2	445	147	296.00	644	285	4
9	2	LW3	567	768	667.50	432	281	3
10	2	RW1	438	428	433.00	641	1036	8
10	2	RW2	527	283	405.00	479	484	4
10	2	RW3	420	433	426.50	209	437	3
11	2	LW1	440	503	471.50	600	683	6
11	2	LW2	677	372	524.50	643	444	5
12	2	RW1	82	159	120.50	582	788	6
12	2	RW2	658	720	689.00	466	642	5
13	2	RW1	357	279	318.00	814	325	5
13	2	RW2	288	439	363.50	580	622	6
13	2	RW3	792	905		716	731	
14	2	RW1	323	631	477.00	428	873	6
15	2	LW1	579	441	510.00	354	348	3
15	2	LW2	1166	618	892.00	468	483	4
15	2	LW3	309	696				
16	2	RW1	625	1092	858.50	489	575	5
16	2	RW2	1022	387	704.50	373	444	4
17	2	RW1	1029	605	817.00	983	514	7
17	2	RW2	288	429	358.50	980	621	7
18	2	RW1	409	521	465.00	2028	536	12
18	2	RW2	998	609	803.50	858	697	7
19	2	LW1	1181	462	821.50	643	504	5
19	2	LW2	532	922	727.00	751	477	6

FUTURE COSME

From Concept to Product

APPENDIX G:

FUTURE COSM

From Concept to Prod

INITIATION DATE:	2019/11/01
COMPLETION DATE:	2019/11/29
STUDY NUMBER:	FCAH163
EVALUATION:	VISIOSCAN VC98
PARAMETER:	WRINKLE REDUCTION (HISTOGRAM)

Table G1: STATISTICAL ANALYSIS OF VISIOSCAN VC98 FOR FCAH163/6606 VS NEGATIVE CONTRO for BASELINE

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between and FCAH163/6705 for BASELINE

H1 -There is a significant difference between and FCAH163/6705 for BASELINE

Chi-Squared Test of Normality

	Column 1
Mean	128.3491
Standard deviation	456.5824
Observations	53

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	8.408715	7
(-1 < z <= 0)	0.341345	18.091285	25
(0 < z <= 1)	0.341345	18.091285	17
(z > 1)	0.158655	8.408715	4

chi-stat	5.251656566
df	1
p-value	0.021929911
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Col
T+	960
T-	-481
Observations (for test)	53
z Stat	-2.075873364
P(Z<=z) one-tail	0.01894021
z Critical one-tail	1.6449
P(Z<=z) two-tail	0.03789642
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between and FCAH163/6705 for BASELINE

INITIATION DATE:	2019/11/01
COMPLETION DATE:	2019/11/29
STUDY NUMBER:	FCAH163
EVALUATION:	VISIOSCAN VC98
PARAMETER:	WRINKLE REDUCTION (HISTOGRAM)

Table G2: STATISTICAL ANALYSIS OF VISIOSCAN VC98 FOR FCAH163/6606 VS NEGATIVE CONTRO for D14

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between and FCAH163/6705 for D14

H1 -There is a significant difference between and FCAH163/6705 for D14

Chi-Squared Test of Normality

	Column 1
Mean	-139
Standard deviation	402
Observations	52

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	8.25036	7
(-1 < z <= 0)	0.341345	17.74964	19
(0 < z <= 1)	0.341345	17.74964	17
(z > 1)	0.158655	8.25036	9

chi-stat	0.377303253
df	1
p-value	0.539050035
chi-squared Critical	3.8415

t-Test: Paired Two Sample for Means

Mean	-117.75
Pearson Correlation	0.129129941
Hypothesized Mean Difference	0
df	51
t Stat	-2.07703717
P(T<=t) one-tail	0.021426744
t Critical one-tail	1.67528485
P(T<=t) two-tail	0.042853489
t Critical two-tail	2.00758377

H0 - data is normally distributed

H1 -There is a significant difference between and FCAH163/6705 for D14

INITIATION DATE:	2019/11/01
COMPLETION DATE:	2019/11/29
STUDY NUMBER:	FCAH163
EVALUATION:	VISIOSCAN VC98
PARAMETER:	WRINKLE REDUCTION (HISTOGRAM)

Table G3: STATISTICAL ANALYSIS OF VISIOSCAN VC98 FOR FCAH163/6606 VS NEGATIVE CONTRO for D28

FUTURE COSME

From Concept to Product

INITIATION DATE: 2019/11/0

COMPLETION DATE: 2019/11/2

STUDY NUMBER: FCAH16

APPENDIX H: DEVIATION

DEVIATION FROM:	NOTE:
SUBJECT 04	LOST TO FOLLOW
SUBJECT 09	NO LW2 ON D28