STUDY ON SYNERGISTIC EFFECTS OF AZELAIC ACID, KOJIC ACID AND HYALURONIC ACID IN MELANIN INHIBITION WITH ZEBRAFISH MODEL

Submitted in partial fulfillment of the requirements for the award of

Master of Science

In

Medical Biotechnology and Clinical Research

By

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CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES
INTERNATIONAL RESEARCH CENTRE

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

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BONAFIDE CERTIFICATE

This is to certify that this project report is the bonafide work of **Ms. SANGAMITHRA E** who carried out the project titled **"STUDY ON SYNERGISTIC EFFECTS OF AZELAIC ACID, KOJIC ACID AND HYALURONIC ACID IN MELANIN INHIBITION WITH ZEBRAFISH MODEL"** under my supervision october 2022 to april 2023.

for

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(DR. R. RAJESH KANNAN)

HEAD OF THE DEPARTMENT
(DR. A. DAYANAMDAN)

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Submitted for Viva voce Examination held on 05.05.2023

Internal examiner

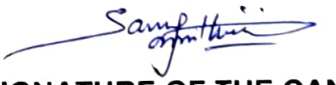
External examiner

5/5/23

DECLARATION

I, Ms. SANGAMITHRA E (41101011) hereby declare that the Project Report entitled **"STUDY ON SYNERGISTIC EFFECTS OF AZELAIC ACID, KOJIC ACID AND HYALURONIC ACID IN MELANIN INHIBITION WITH ZEBRAFISH MODEL"** was successfully done by me under the guidance of Dr. R. Rajesh Kannan submitted in partial fulfillment of the requirements for the award of Master of Science Degree in Medical Biotechnology and Clinical Research.

DATE: 05.05.2023
PLACE: CHENNAI



SIGNATURE OF THE CANDIDATE

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ABSTRACT

Zebrafish is widely used as a model for melanin studies because of its high fecundity, its ability of tail regernation and having visible melanin pigment on its body. Evaluation of anti-melanin activity of several depigmenting agents using zebrafish embryos were increasingly reported. Melanin is a salient complex polymer for protecting skin, while excess of it may cause some demerits to skin pigmentation. Melanin inhibition can be achieved by targeting the tyrosinase pathway which is responsible for the melanin production. Azelaic acid is a well known compound as a tyrosinase inhibitor where as kojic acid has the ability of skin whitening. Hyaluronic acid has the catalytic properties of enhancement and hydration. More successful development whitening skin care products rely on the use of effective whitening or depigmenting ingredients that inhibit melanogenesis in melanocytes. Synergistic use of the compounds have higher ability to inhibit melanin than the single compound usage.The purpose of the study is to determine the favorable ratio of these compounds and study the effects of the combination with the zebrafish model and achieve transparency, which could be used for the future studies with this model.

Keywords: Zebrafish, Melanin inhibition, Azelaic acid, Kojic acid, Hyaluronic acid

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AzA - Azelaic acid

Ko- Kojic acid

HA- Hyaluronic acid

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CHAPTER 1
INTRODUCTION

1. BACKGROUND

In this present era skin enhancement is most individual's prior concern as the recent global survey states that 94% of 14,584 people interested in improvising their skin and its quality(humprey, 2021). Melanin is a salient complex polymer for protecting skin, while excess of it may cause some demerits to skin pigmentation (Danni Feng, 2021). Tyrosinase is a key target for the discovery and screening of the novel inhibitors because of its prominent role in the melanogenesis through tyrosine pathway. A delicate and highly interesting relationship which exists between antioxidant defence systems and melanogenesis is assosiated with the ROS scavenging which thereby increases the effectiveness of the antioxidants in scavenging free radicals while tyrosine inhibitors work ultimately leads to the reduction of melanin productionYan Wang, 2018). Melanogenesis is a process that results in the production of melanin, the pigment responsible for coloring human skin, eyes, and hair. This process involves several chemical reactions and enzymatic catalysis, with tyrosinase, TRP-1, and TRP-2 being the key enzymes involved in melanin synthesis. Tyrosinase, in particular, is essential as it catalyzes the rate-limiting step in melanin synthesis, and suppressing its activity is a major approach for developing melanogenesis inhibitors (Thanigaimani Pillayar, 2017).

2. AZELAIC ACID

Azelaic acid (AzA) is a dicarboxylic acid that is produced by the commensal yeast, Malassezia furfur, which is commonly found in the skin flora of humans and animals. In addition to this, AzA is also present in various food sources such as wheat, barley, and rye, which results in exposure to the acid through dietary intake. Studies have indicated that AzA is safe for human use, as it lacks any acute or chronic toxicity, mutagenic properties, or teratogenic effects (James Q Del Rosso, 2017). Azelaic acid is often used in the treatment of acne at a concentration of 15% in gel and 20% in cream formulations. Because it does not function well, it is not advised to use concentrations lower than 10%. While using more than 10% is considered to be a form of medical treatment (E Lusianti, 2018). AzA has been shown to be well tolerated in various clinical trials and to be generally successful in

treating a variety of causes of acne (M A Sieber, 2014). For a variety of dermatological disorders, azelaic acid, either alone or in combination with other treatments, may be an efficient first-line or alternative therapy that is both well-tolerated and safe (Tamara Searle).

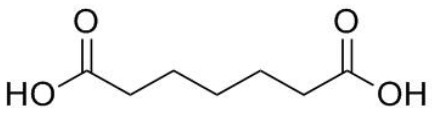

***Fig 1.1** azelaic acid chemical structure*

3. HYALURONIC ACID

The use of hyaluronic acid (HA), sometimes called hyaluronan or hyaluronate, and its derivatives in cosmetic compositions. N-acetylglucosamine and D-glucuronic acid combine to form HA, a glycosaminoglycan that was first isolated from the vitreous humour of the eye and later found in a variety of tissues and fluids, particularly in the synovial fluid and articular cartilage. Involved in a wide range of biological activities, including cell differentiation, embryological development, inflammation, wound healing, etc., it is found in all vertebrates, including humans. HA offers several advantages over other skin-regeneration agents, including moisturising and anti-aging properties. The biological activity and skin penetration of HA are influenced by its molecular weight (Anca Maria Juncan, 2021).

The possible permeation-enhancing functions of HA for the skin were several of its delivery mechanisms, including receptor-based delivery pathways, skin hydration, hydrophobic interaction with stratum corneum, bioadhesive qualities, and viscoelastic properties. Different delivery systems, such as hydrogel, nanoemulsion, microemulsion, prodrug, microneedle, and liposome/hyalurosome, were designed using HA with different molecular weights and chemical modifications in order to achieve the best delivery efficacy for bioactive compounds at different target layers of the skin. Applying the Franz Cell Diffusion

method in vitro and/or in vivo animal models, delivery effectiveness has been assessed (Jieyu Zhu, 2020).

Catabolism of hyaluronic acid can take either chemically or enzymatically. The following hyaluronidases are distinguished: The enzyme HYAL1-, which is connected to lysosomes, converts HA into tetrasaccharides. High molecular mass HA is broken down by HYAL2 into molecules that are 20 kDa in size. We don't know anything about the enzyme HYAL3, PH-20 is found in sperm, and HYALP1. B-glucuronidase, N-acetyl-glucoaminodase, and exoglycosidases are involved in the enzymatic activities. The specificities of the enzymes, as emphasised by Vopli, have been the subject of recent research. Because of this, despite their extremely low abundance, they are quite active. They also require particular extraction conditions, and studying them requires a lot of work. In turn, reactive oxygen species are linked to chemical deterioration. The products are shorter, with a length of 4-6 saccharide chains. Superoxide dismutase is prevented by reactions connected to the breakdown of the smaller HA pieces (Natalia M Salwowska, 2016). Karl Meyer and John Palmer first identified a novel polysaccharide in the vitreous humour of cattle in 1934. They discovered that the material contained an aminosugar and a uronic acid, and they gave the polysaccharide the name "hyaluronic acid" by combining the words hyaloid (vitreous) and uronic acid (Long Liu, 2011).

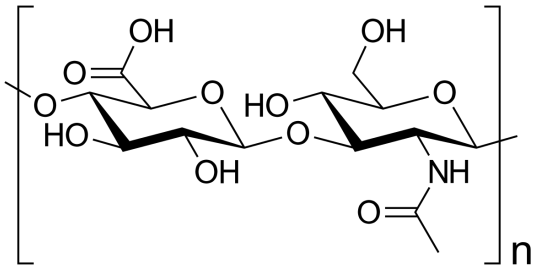

Fig 1.2 *hyaluronic acid chemical structure*

4. KOJIC ACID

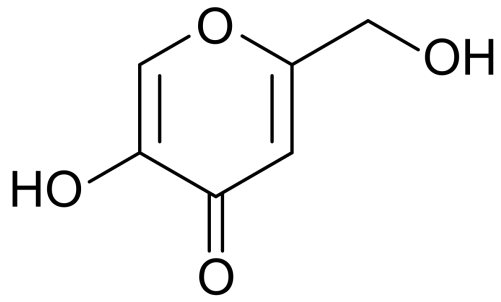

Fig 1.3 *kojic acid chemical structure*

A number of microorganisms utilise carbohydrate sources to create the kojic acid, 5-hydroxy-2-(hydroxymethy1)-4H-pyran-4-one, in an aerobic process. The mycelia of Aspergillus orgzae, which are cultivated on steamed rice, are where kojic acid was initially discovered as a crystalline material. One of these is kojic acid. It is indicated how the atoms of kojic acid's -pyrone ring are ordinarily numbered. Kojic acid crystallises as prismatic, colourless needles. Water, ethanol, and acetone are the only liquids in which kojic acid is easily soluble; ether, ethyl acetate, chloroform, and pyridine are the only liquids in which it is sparingly soluble. It was refined through recrystallization from acetone, ethanol-ether, and methanol-ethyl acetate as well as sublimation at reduced pressure and 150–200°C. When other organic acids are not present, the amount of kojic acid in a solution has been determined volumetrically by titration with a standard, diluted alkali, using Alizarin Orange R37 or phenolphthalein as an indicator, and colorimetrically by measuring the intensity of the red colour produced with ferrous and ferric chloride (Andrew Beelik, 1956).

Kojic acid is used at concentrations ranging from 0.1% to 2%, with the highest concentration being used in face and neck creams, lotions, and powders, according to a survey conducted by the Personal Care Products Council on current use concentrations (Chirstina L Burnett, 2010). Kojic acid is currently used primarily as the primary component of excellent skin lightening cosmetic creams, where it works to prevent the production of pigment by the skin's deep cells. Currently, this acid is also widely used in the cosmetic industry as a skin protective lotion because the incidence of skin cancer is rising quickly due to exposure to high ultraviolet radiation from sunlight. In order to control lightened freckles and

age spots, it is typically used in combination with alpha-hydroxy acid in the formulation of skin whiteners.Asia has outlawed the use of hydroquinone for cosmetic purposes, and the US Food and Drug Administration (FDA) has identified it as a compound that may cause cancer. Because hydroquinone bleaches and may harm skin, there has been a significant increase in the use of kojic acid as a substitute in cosmetic products. Kojic acid and its complexes with manganese and zinc may also be used as radioprotective substances, particularly against γ-ray (Rosfarizar Mohammed, 2010).

5. MELANIN INHIBITION

Skin melanin pigments play an important role in determining skin color and are synthesized by large dendritic cells known as melanocytes at the junction of the epidermis and dermis. Melanocyte tyrosinase is a key enzyme in the synthesis of melanin pigment. Melanocytes transfer melanin pigment to neighboring cells such as keratinocytes. The production and transport of melanin is increased by factors such as UV rays, hormones, and chemicals, leading to the development of skin darkening, age spots, freckles, melasma, and other hyperpigmentation disorders. It is very popular and is used to lighten the skin and treat freckles and hyperpigmentation. More successful development whitening skin care products rely on the use of effective whitening or depigmenting ingredients that inhibit melanogenesis in melanocytes (Majmudar, 1998).

Uncontrolled accumulation of melanin is a serious social problem not only for women but also for men, causing hyperpigmentation disorders such as freckles, chloasma, and pigmented acne scars. Synergy is widely used in medicine, and its potency makes pharmaceutical applications more valuable (You Cheng Hseu, 2015). From the wide range of study it is clear that the above mentioned compounds azelaic acid, kojic acid and hyaluronic acid has the effects to inhibit melanin thus improving the goodness of the skin.

6. ZEBRAFISH (*Danio rerio*)

6.1 Taxonomy and nomenclature

Genbank common name: zebrafish

Rank: species

common name(s): leopard danio, zebra danio, zebra fish
Kingdom: Animalia
Phylum: Chordata
Phylum: Chordata
Superclass: Gnathostomata
Class: Actinopterygi
Order: Cypriniformes
Family: Cyprinidae
Genus: Danio
Species: Danio rerio

6.2 Zebrafish as melanin study model

Zebrafish are a new model organism used to study their high fecundity, the development of visible melanin in melanophores (mammalian melanocytes) from 24 h after fertilization, and pigment disorders through conserved melanogenesis pathways (Sam J Neuffer, 2022). Evaluation of anti-melanin activity of several depigmenting agents using zebrafish embryos were increasingly reported. Application of the zebrafish depigmentation assay supported by the effects of well-known bioactive compounds such as kojic acid and arbutin on the embryo depigmentation. Kojic acid's association with many past and present dates with arbutin depigmentation assays in vitro (i.e., melanocytes) and in vivo (i.e., mice) are currently improving investigation and understanding using zebrafish embryos (Ahmad Firdaus B. Lajis, 2018). Moreover, zebrafish are superior to rodent models in several ways for the study of vertebrate development and disease. A single clutch can include hundreds of embryos of zebrafish, and the optical purity of the growing embryo enables for real-time observation at the organism level (Tae Young Choi, 2021).

As for now, no work is done on synergistic effects of the triple compounds azelaic acid, kojic acid and hyaluronic acid and studied their synergistic effects on the zebrafish model. Further, treatment of the combination of three compounds on the zebrafish embryo is yet to be studied.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Studies on azelaic acid

Schulte BC et al,. 2015 stated azelaic acid as a complicated chemical with a wide range of functions. The latter have anti-inflammation and anti-infective properties. Additionally, the substance prevents epidermal melanogenesis and follicular keratinization. In addition they stated that azelaic acid has been used as a management strategy in a wide range of disease states and dermatological illnesses due to the large variety of biological activities.

Cunliffe WJ et al,. 1989 reported that in a series of studies utilising 20% azelaic acid cream as a treatment for acne, it was discovered that the medication considerably reduced inflamed lesions after 1 month and non-inflamed lesions after 2 months when compared to its placebo. Also sebum excretion rate remained unchanged, however after a month, there was a noticeable drop in the amount of free fatty acids in skin surface lipid, from 15.9 to 10.5%. After one month, the density of the follicular Micrococaceae greatly decreased, and after two months, the density of the follicular Propionibacterium species also significantly decreased. They presented that about 2,500 and 44 fold reductions were the end results, respectively.

Zoe Diana Draelos et al,. 2015 Did the work on the creation of an AzA foam formulation with a higher lipid content has the goal of giving doctors and rosacea patients more options for treatment. However, patients who use topical therapies rank messiness and ease of application as two of the most crucial factors affecting quality of life. They stated majority of topical dermatologic treatments are currently administered in conventional formulations such as creams or gels. Foam formulations may provide advantages in this area; the simplicity of usage may reduce needless manipulation of inflamed skin and increase user satisfaction. They have suggested that the foam formulation combines the proven therapeutic capabilities displayed by AzA gel 15% with the good tolerability and acceptability of a lipid-containing foam formulation in order to address the unique needs of the dry and sensitive skin in rosacea.

2.2 Studies on kojic acid

Christina L. Burnett et al,. 2010 presented that however kojic acid is slowly absorbed through the human skin into the circulatory system, It probably does not reach the systemic levels at which these effects have been observed. They said available human sensitization data support the safety of his 2% concentration of kojic acid in leave-on cosmetics, suggested that a 2% limit may be appropriate. However, a depigmentation study with kojic acid in black guinea pigs found statistically significant skin lightening at a concentration of 4%. In the same study, they noted that a 1% kojic acid concentration did not result in different skin lightening than vehicle controls. Also, Kojic acid does not appear to damage melanocytes, and the skin lightening effect at 4% is probably due to tyrosinase inhibition. Although reversible, the panel considers tyrosinase inhibition to be an adverse effect with a NOEL of 1%. They concluded that the expert panel therefore states that kojic acid should only be used in cosmetics in concentrations up to 1%.

C. I. Wei et al,. 1991 concluded from their work that the kojic acid was toxic to Chinese hamster cells at high doses (>9 mg/mL). Observed morphological cytopathic effects included cell rounding and loss of nucleolus definition. From cellular protein loss, the Chinese hamster cell line kojic acid level was determined to be 10.86 + 3.86 (standard deviation) mg/ml medium.

Majid Saeedi et al,. 2019 stated that the most important advantage of KA is its wide range of applications is in the cosmetic and medical industries. And added that it functions as a whitening ingredient in skin lightening creams, skin lotions, bleaching soaps, and dental and medical care products. Therefore, they concluded that more clinical studies are needed for the design and development of new products based on KA.

Brtko J. et al,. 2004 noted that Tyrosinase is the key enzyme that plays a rate-limiting role in the manufacture of the skin pigment melanin, is known to exhibit catecholase activity that is inhibited by kojic acid. Due to the reactivity of kojic acid with metals, melanocytes treated with it become nondendritic and have less melanin. And resulted that kojic acid is regarded as a potent skin-lightening/depigmenting ingredient for use topically in skin care products.

2.3 Studies on hyaluronic acid

Richard D. Price et al,. 2007 has stated that the purified Hyaluronic Acid was used to heal a burn in 1968, which is believed to be the year that HA was first used therapeutically. And added, since then, it has been used in practically all medical specialties; for example, physicians are already familiar with the use of hyaluronidase to improve the absorption of fluid under the skin and to treat extravasation injuries.

Barrie Fong Chong et al,. 2004 has confessed that due to interactions with the solvent, internal hydrogen bonds, and -glycosidic bonds, HA exhibits conformational stiffness in physiological solution. As a result, in solution, HA adopts an enlarged coil shape that fills a sizable domain. Even at low concentrations, HA solutions behave in a very non-Newtonian, gel-like manner because to this and their high molecular weight. They claimed that three categories of conduct exist: semi-dilute, concentrated, and diluted. Each molecule behaves almost Newtonian and operates as a suspended particle in the diluted region. From their study they concluded that above 1.5%, we move into the concentrated region, where networks start to form and eventually result in stable gels.

Peisong Zhai et al,. 2020 discussed that the hyaluronic acid-based bone regeneration scaffolds are more bioactive and compatible with biomimetic techniques, according to recent research. Moreover, particularly sulfated HA, may stimulate cell behaviour modification through a number of signalling channels as a matrix component, resulting in more expedient and desired bone production. Also, Based on HA, rigid structures or colloids are created for scaffolds and carriers. When combined with other materials, HA, which is a hard scaffold material, may change the scaffold's morphology and enhance mineralization, making it more appealing and useful for bone regeneration.

2.4 Synergistic studies on the compounds

Wioletta Baranska-Rybak et al,. 2021 concluded that in individuals with aging-related skin issues, combined therapy based on HA fillers and individualised skincare with Universkin^{TM} products show encouraging outcomes. The ability for physicians to blend topical products can help them tailor it to the needs of each patient's skin. Also confessed that the best therapeutic outcome would therefore be attained as a result of it.

Iva Dolečková et al,. stated that together, the two biologically active chemicals LMW HA and α-linolenic acid combined into one molecule provide several benefits. In contrast to the α-linolenic acid alone, this system is able to produce micelle-like structures that get deeply into the skin and skin cells where they effectively suppress the process of melanogenesis. The chemical significantly lightens pigmented spots in both Asian and Caucasian skin types in vivo and diminishes them.

Alfredo Martinez-Gutierrez et al,. 2014 stated that when kojic acid and azelaic acid are combined, a potentiation effect is seen. While azelaic acid seldom suppresses melanin formation on its own, the combined impact is larger than the individual suppression by kojic acid (22% vs. 16%). They presumed that action can be explained by the likelihood that arbutin and azelaic acid facilitate the inhibition of kojic acid and -lipoic acid, respectively, enhancing the inhibition of melanin.

CHAPTER 3
AIM AND OBJECTIVE

AIM:

To study the synergistic effects of azelaic acid, kojic acid and hyaluronic acid in combination treatment on zebrafish model for melanin inhibition and regeneration..

OBJECTIVE:

- ✧ To find the concentration ratio of the selected compounds with melanin inhibition in zebrafish embryo.
- ✧ To estimate the melanin content in the treated zebrafish embryos.
- ✧ Observation and study of the zebrafish tail regeneration with the derived ratio.

Chapter 4
METHODOLOGY

4.1 Zebrafish embryo maintenance and treatment

Zebrafish embryos were brought commercially and was maintained in E3 (ethylene blue) media as it limits the possibility of fungal contamination. The incubation room was maintained at 27-28 degree celsius.

4.1.1 E3 media preparation

PREPARATION

To prepare 100 ml 60 X- E3 media stock

Nacl-1.74g

KCl- 0.08g

CaCl2- 0.29g

MgCl2- 0.489g

PROCEDURE

i. The above mentioned components were dissolved in 100ml distilled water to make 60X stock medium.
ii. The stock medium is adjusted to 7.2 ph with 1% NaOH solution.
iii. To prepare 1X medium of E3 media, 1ml from 60X stock medium solution is added to 59ml of fresh water to make 60 ml of 1X
iv. To the 1X solution a very minimal pinch of 1% ethylene blue is added.

4.2 Compound combination preparation

Azelaic acid 40% powder, kojic acid pure powder and hyaluronic acid pure powder are bought commercially from banglore fine chemicals industry.

With the selected three compounds azelaic acid, kojic acid and hyaluronic acid about 36 combinations were formed with the ratio of AzA : ko: hyA were azelaic acid is taken in 15%, 18% and 20%; kojic acid is taken in 1%, 1.5% and 2%; hyaluronic acid is taken in 1%, 1.5% and 2% as higher, medium and lower concentrations and made 36 combinations with it.

4.3 Zebrafish embryo treatment

Zebrafish embryos were suspended in two 24 well plates with 20 embryos in each well and the excess water is removed using a pipette and the created combinations were added to the each wells respectively containing 2 ml in each well. Also, 2 wells were kept as control to compare with the treated embryos. The wells were let to incubate in room temperature and the results were observed on the basis of continuous days as day 1 with 24 hours and day 2 with 48 hours of incubation. After the observation, the beneficiary combination is selected from the 36 samples and with that combinations which has the effective positive results is taken for the further works.

4.3.1 Zebrafish larvae treatment and observation

The effective combination percentage, AzA 15% : KO 1.5% : HYA 1% is treated to the commercially bought zebrafish embryo.

MATERIALS

Sterile Petridish- 2nos
Azelaic acid powder- 0.555g
Kojic acid powder- 0.900g
Hyaluronic acid powder- 0.300g
E3 media- 28.245ml
Pipettes
Live zebrafish embryos

PROCEDURE

i. One petridish is labelled as the control and other as treatment.
ii. Control petridish is filled with 30ml E3 media and about 200 embryos were suspended to grow in it.
iii. Azelaic acid, kojic acid and hyaluronic acid is dissolved in E3 media as per the above mentioned measures to make up to 30 ml and filled in the treatment petridish and 200 embryos were suspended to grow in it such that as in control.

4.4 Melanin content estimation assay

MATERIALS

Centrifuge tubes - 6 nos
Pipette
1M tris buffer

1M NaOH

Homogenizer

Dry water bath

Nano drop

Vortex

PROCEDURE

i. Two Sterile centrifuge tubes are taken and labeled as control, treatment, control supernatant and treatment supernatant.
ii. Twenty larvaes were suspended in control centrifuge tube from the control petridish.
iii. Twenty larvaes were suspended in treatment centrifuge tube from the treatment petridish.
iv. Excess water is removed from both the tubes with larvae and 200 microlitre of 1M Tris is added and homogenized with homogenizer and vortexed for 1 - 2 minutes.
v. The supernatant is carefully separated and suspended in the tubes control supernatant and treatment supernatant from the respective tubes.
vi. To the control and treatment tube which is with the pellet, 1 ml of NaOH is added.
vii. The four centrifuge tubes are kept in the dry water bath for 1 hour at 95 degree celsius.
viii. After 1 hour the tubes are taken from the dry bath and the protein and melanin content is determined using the nanodrop protein estimator and optical density is also noted.

This process is done on the day 1, day 3 and day 5 after the treatment of the embryos.

4.5 Zebrafish adult regeneration

Zebrafish adults were bought from commercial store and maintained in the laboratory. 20 zebrafish adults were kept in fresh water and maintained in the room temperature for a period of 1 week for acclimatization. The fishes were fed 3 times a day.

Initially, the fishes were segregated into 2 tanks, control and treated. The solution for the treatment tank is made by dissolving Azelaic acid-2.5g; Kojic acid acid-0.44g and 0.156g of hyaluronic acid in 200 ml fresh water, where the measures of the components were in the ratio of 15%: 1.5%: 1%. The tail of all the 20 fishes were amputated under a stereo microscope with a sterile surgical blade and a image of it is taken for each of the fish and stored. The observations were made in the day 3, day 5 and day 9 after the tail cutting.

4.5.1 Calcein stain preparation

To evaluate the growth of the zebrafish bone in its tail, calcein is used as a fluorescent stain.

PROCEDURE

i. For calcein stain stock, 0.2% calcein solution with deionized water is prepared that is 2g calcein powder in 1000ml deionized water.
ii. The ph is adjusted to 7 with NaOH and HCl.

4.5.2 Tricane preparation

For the anasthesia and euthanasia of laboratory zebrafish, Tricane methanesulfonate(MS222) is widely used as freshly preapred solution.

MATERIALS

Tricane powder- 400mg

Double distilled water- 97.9ml

1m tris buffer- 2.1ml

PROCEDURE

Tricane stock

i. Prepare 1M tris buffer by adding 12.114g of tris to 100ml distilled water.
ii. Add tricane powder, 1X tris to the double distilled water.
iii. Adjust the ph to 7 using NaOH and HCl.
iv. Store the prepared stock in the freezer at -18 degree celsius.

Tricane working solution

i. Add 4.2 ml of tricane stock solution to 100ml of tap fresh water.

ii. Prepare tricane working solution freshly everytime at use to maintain the efficacy of the tricane.

4.5.3 Regeneration study procedure

i. On the day 3 after the zebrafish tail is cut the fishes from the control tank and the treatment tank were observed under stereomicroscope.
ii. The fishes were suspended in the prepared calcein solution for about 15-20 minutes.
iii. The stained fishes are washed 3 to 5 times with tap fresh water to destain the fishes.
iv. After destaining fishes are kept in tricane working solution one by one for 1 minute to make still without shaking and pictured in stereomicroscope. The growth of the fin is noted in the fluroscence images taken.
v. After taking images, fishes are again let in the control tank and treatment tank respectively.
vi. The same process is repeated on the day 5 and day 9.

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Zebrafish embryo treatment

With the calculated 36 combination of azelaic acid, kojic acid and hyaluronic acid having three concentration each treated to the twenty embryos per combination shows delayed growth and chorion (acellular membrane) issues in the concentrations where the hyaluronic acid is 1.5% and 2% but, in the lowest concentration 1% of hyaluronic acid good growth and healthy embryos are observed.

Fig. 5.1 *day 1 observation of embryos treated with 36 combinations.*

In the wells were the hyaluronic acid is in lower concentration that is 1% in alone as well as in combination shows healthy development whereas in the higher concentration blood clot, embryo chorion damage and deformatives are seen in the day 1 observation (fig. 5.1). Delayed growth is observed in higher concentrations of hyaluronic acid and kojic acid.

On the day 2 observation the larvae were not formed clearly in the combinations were hyaluronic acid concentration were 1.5% and 2% and also where the kojic acid is either high or low that is 1% and 2%. It is clear that in the higher concentration of azelaic acid 20% the embryo have not developed whereas in

lower concentration of azelaic acid alone and in combination showed good development of embryo without any of the blood clot.

In the combination where azelaic acid: kojic acid: hyaluronic acid is 37:60:20 that is 15%: 1.5%: 1% and in the combination of 20%: 2%: 1% (fig. 5.2), healthy larvae without any blood clot or deformatives are seen. These combinations as taken as the favorable concentration for the further works. Moreover, in 37:60:20 that is 15%: 1.5%: 1% combination transparency of the larvae is observed.

Fig 5.2 *Day 2 observation of the treated embryos with the calculated 36 concentrations.*

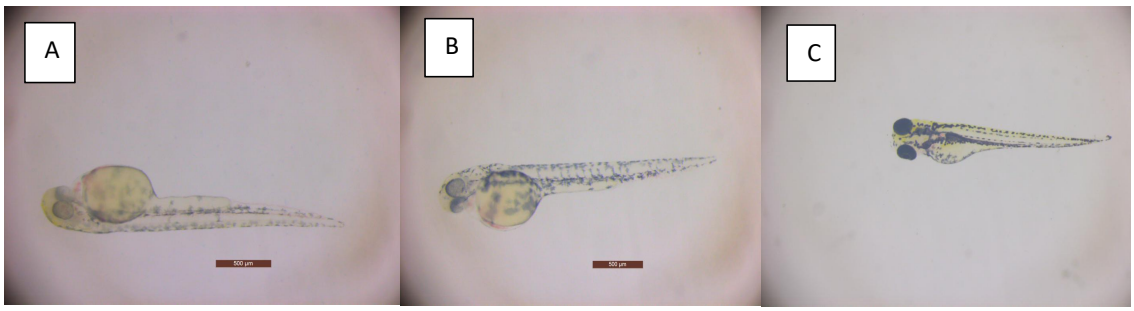

Fig 5.3 (A) *Day 2 treated larvae with combination 20%: 2%: 1%;* ***(B)*** *Day 2 treated larvae with combination 15%: 1.5%: 1%;* ***(C)*** *Control larvae which is not treated with any compounds.*

In fig 5.3 it is clearly seen that in A and B is slightly transparent when compared to the control. The spots on the larvae are lighter when compared to that of control larvae.

5.2 Embryo treatment with derived concentration

After, the embryos are treated and observed on the next day 1 transparency is seen in the treated larvae compared to the control larvae. On the third day the transparency in the treatment larvae is more improved. On the fifth day of observation the treated fishes are found little darker that that of the control fishes. Prolonged exposure of the larvae in the combination suspended shows a reversing effect of the melanin.

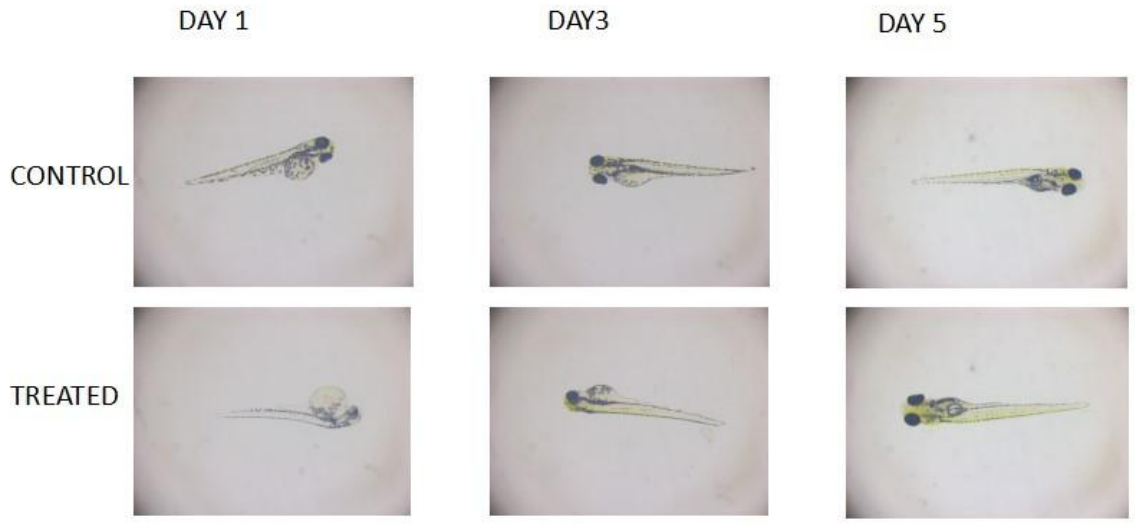

Fig 5.4 *Control and treated larvae on the day 1, 3 and 5*

By using image j software the area of the melanin pigment on the zebrafish larvae is determined (table 5.1). It shows the melanin pigment is increased from day 3 and higher in day 5 when compared to the control.

Table 5.1 *area of melanin pigment on zebrafish larvae*

Day	Control mm^2	Treated mm^2
1	37149	23030
3	33939	27648
5	33327	43966

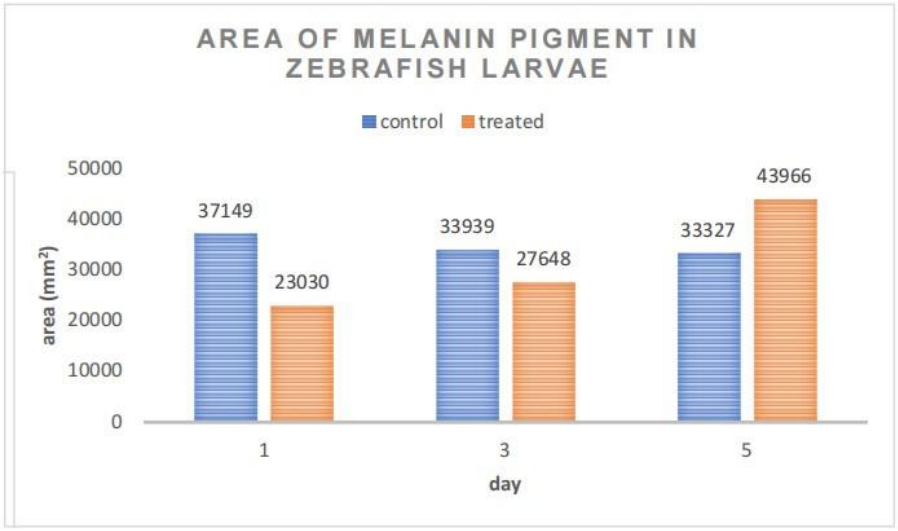

Fig 5.5 *Area of the melanin pigment in zebrafish larvae in mm^2*

5.3 Zebrafish adult treatment with the favorable concentration

Ten fishes were taken as control and tail cut under stereomicroscope and another ten fishes tail cut for treatment.

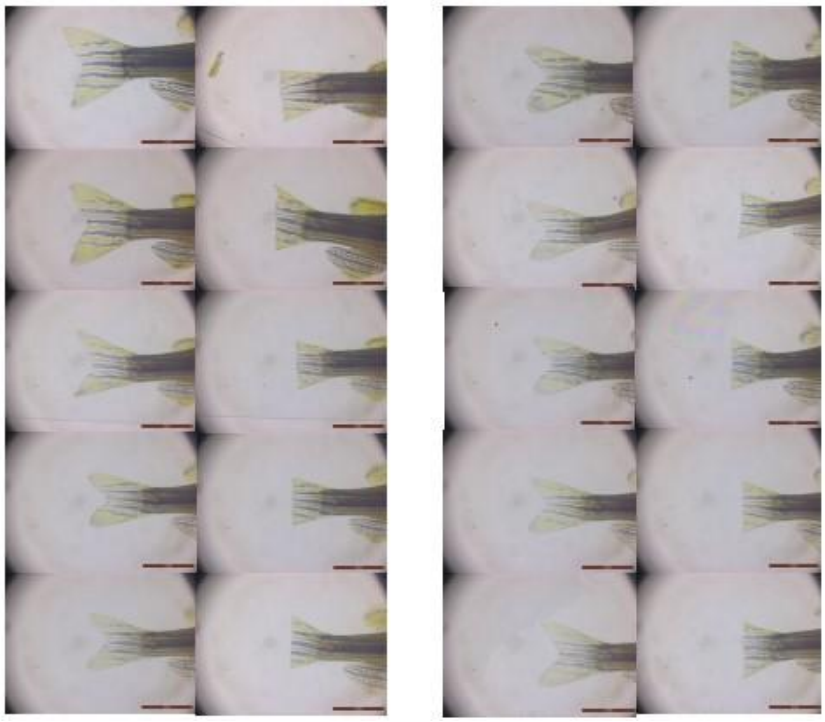

Fig 5.6 *Day 0 adult fishes for control- tail cut before and after*

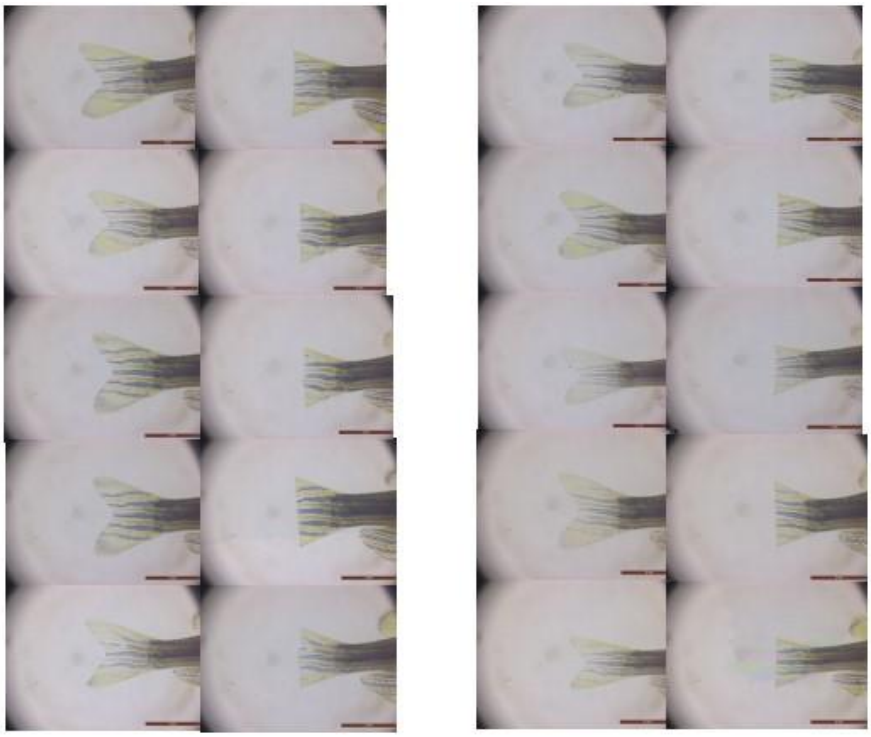

Fig 5.7 *Day 0 adult fishes for treatment tail cut before and after*

On the day 5 observation of the control and treatment fishes, one of the fishes from the 10 fishes of the treatment set died. The left over 9 fishes from the treatment and 10 fishes from control were observed and there is no delayed growth is seen.

The tail of the treated fishes were well grown as in control. The calcein stained well and showed the regenerated cartilage in the tail of the fishes.

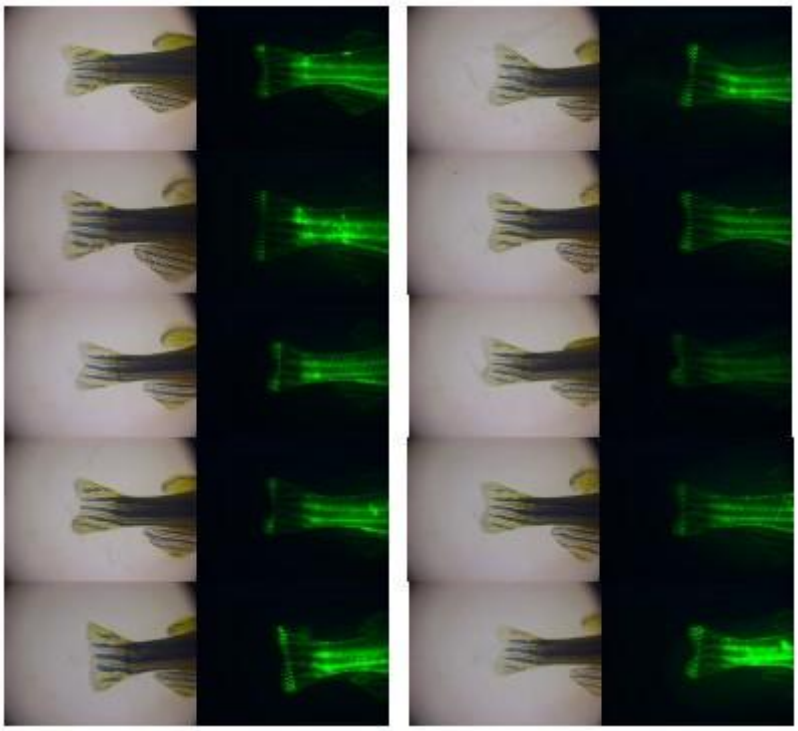

Fig 5.8 *Day 5 regeneration of the tail of control fishes*

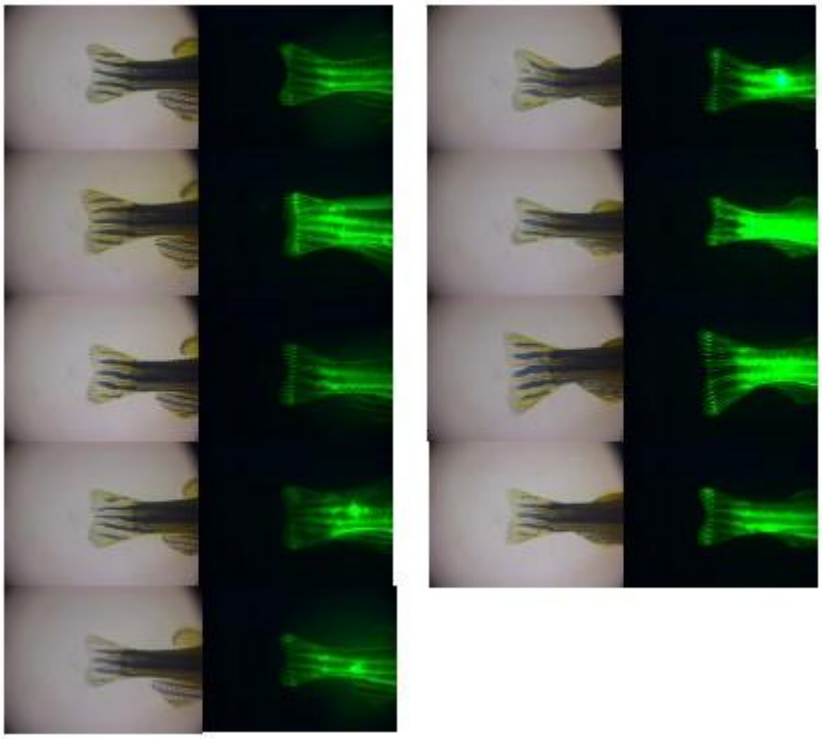

Fig 5.9 *Day 5 regeneration of the tail of treatment fishes*

With the tail regeneration study it shows good growth on the beginning stage day 1 and 3 and on the day 5, the growth of the tail is slightly delayed when compared control.

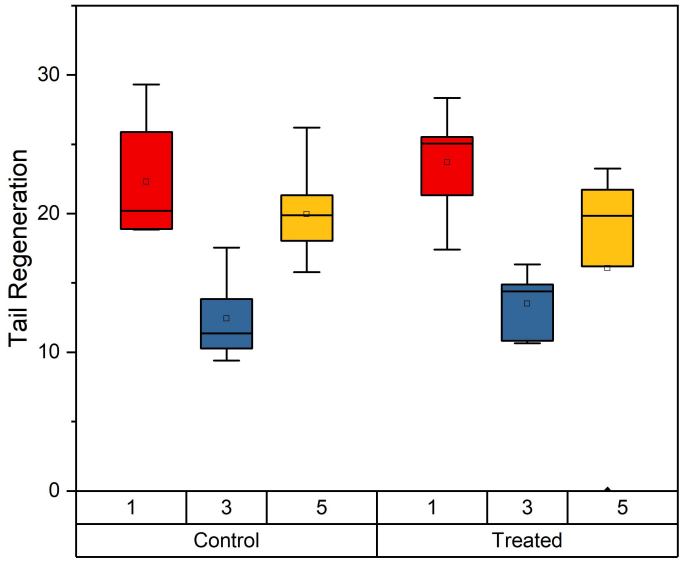

Fig 5.10 *Tail regeneration mean area value*

CHAPTER 6
SUMMARY AND CONCLUSION

In this present study we have determined the effective ratio of concentration with the combination of the three compounds azelaic acid, kojic acid and hyaluronic acid is 15%: 1.5%: 1%. Other that this ratio shows chorion damage, blood clot and delayed growth in the zebrafish embryo. Initially larvae which is exposed to the found ratio showed transparency and low melanin content and when in a prolonged exposure of the larvae in this combination for more that 3 days shows a reversible effect of the melanin pigment. Regeneration studies for a period of 1 week shows healthy growth of the tail in the treated, the growth of the tail is slightly delayed on the fifth day. In conclusion the combination of the three compounds in the ratio 15%: 1.5%: 1% can be used to reduce the melanin content thus improving transparency, but when used for a prolonged period the effect of the ratio would be reversed by increasing the melanin content.

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