

PREPARATION OF SKIN LIGHTENING CREAM FROM KOJIC ACID EXTRACTED FROM FUNGI *A.oryzae*

(Project – Report)

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

V. DAKSHNI (38230008)



DEPARTMENT OF BIOTECHNOLGY

SCHOOL OF BIO & CHEMICAL ENGINEERING

SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade “A” by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **V. DAKSHNI (38230008)** who carried out the project entitled **“PREPARATION OF SKIN LIGHTENING CREAM FROM KOJIC ACID EXTRACTED FROM FUNGI A.oryzae”** under our supervision from October 2021 to March 2022

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DECLARATION

I **DAKSHNI .V (38230008)** hereby declare that the Project Report entitled **“PREPARATION OF SKIN LIGHTENING CREAM FROM KOJIC ACID EXTRACTED FROM FUNGI *A.oryzae*”** done by me under the guidance of , **Chennai** is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE:

A handwritten signature in blue ink, appearing to read 'Dakshni V', is written over a horizontal line.

PLACE: Chennai

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to Board of Management of **SATHYABAMA** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

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ABSTRACT

Skin brighteners are a kind of therapy methods that can be used for the treatment of skin disorders caused by hyperpigmentation. They may inhibit the biosynthesis of melanin using tyrosinase inhibitors. Melanin is a pigment that is produced inside the melanocytes. When these pigment productions are more than usual situation or their distribution are not normal, development of skin hyperpigmentation occurs. The use of chemical products to reduce skin hyperpigmentation by several mechanisms such as reducing the concentration of melanin is known as whitening of the skin. Nowadays, brightening the skin is one of the most common procedures to improve the hyper pigmented parts of the skin. *Aspergillus oryzae* extract is used as a skin-lightening agent, making it effective to combat hyperpigmentation. One research study also suggests that it contains antimicrobial activities and can help improve the effects of ageing and acne-prone skin. Kojic acid is a fungal metabolic product produced by a few species of *Aspergillus*, especially by *A. oryzae* extract, kojic acid skin lightening cream and its derivatives are used to block the formation of pigment by melanocytes as one of the most popular lightener in cosmetic products

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INTRODUCTION

1.1. Overview

Skin discoloration can be caused by various factors, such as excessive exposure to sunlight, aging and hormonal imbalance during pregnancy, or taking some medications. Kojic acid is a fungal metabolite produced by a few species of aspergillus, especially *A. oryzae*, which is the Japanese common name for koji. This acid is a by-product of the fermentation process of rice, which is used in the production of Japanese rice wine. However, it is also very popular for its use in health and cosmetics. Kojic acid (KA) is an organic acid that covers almost all sectors, acting as an anti-bacterial, anti-fungal, anti-melanosis and anti-sealing agent. In general, kojic acid and its derivatives are widely used in the cosmetics and pharmaceutical industries. Kojic acid (KA) is a natural metabolite produced by fungi that has the ability to inhibit tyrosinase activity in melanin synthesis. KA and its derivatives are used in skin creams, lotions, soaps and dental products as anti-oxidant, anti-proliferative, anti-inflammatory, radio protection and skin lightening agents. KA has the ability to act as a UV preservative, suppressing hyper pigmentation in humans and inhibiting the formation of melanin, due to its tyrosinase-inhibiting action. This will help in treating the acne caused by bacteria in the skin. This can reduce the scars of acne that have not yet disappeared. Kojic acid also has anti-fungal properties. This increases their effectiveness. It is effective in treating yeast infections, candidiasis and fungal infections of the skin such as ringworm or athlete's foot. Kojic acid inhibits the growth of bacteria, fungi and viruses. It inhibits the catabolic activity of tyrosinase, which regulates the ratio and is an essential enzyme in the metabolism of skin pigment melanin. By applying Kojic Acid skin lightening cream topically, you can start seeing results within two weeks. You may find more results or faster results. Kojic acid effectively removes dead skin cells and reveals new, glowing skin.

1.2. DESCRIPTION OF SELECTED SPECIES & PLANTS

Aspergillus oryzae

Division:	Ascomycota
Class:	Eurotiomycetes
Order:	Eurotiales
Family:	Trichocomaceae
<u>Rank:</u>	Species
Kingdom:	Fungi
Division	Ascomycota

General information

A. oryzae is an aerobic fiber fungus, and belongs to the genus Flavia, a subfamily of the genus Aspergillus, known as the A. flavus group. Aspergillus SECTION Flavi contains industrially important species such as A. oryzae, as well as agro- and medically important fungi such as A. flavus and A. parasiticus, which produce a powerful carcinogen called aflatoxin. Taxonomically, A. oryzae is closely related

to *A. flavus*, *A. parasiticus* and *A. sojae*, and has long been used for shoyu fermentation. Despite such close contact, *A. oryzae* and *A. sojae* never produce aflatoxins and are used in the production of fermented foods. Therefore, it is important to accurately differentiate between these four species, although recent taxonomic studies of *Aspergillus* division flywheel have some controversial features. *A. Oryzae* are isolated from soil and plants, especially rice. *A. Orissa* is a naturally occurring and professionally named paddy, Orissa cultivated on *Sativa*. *A. Orissa* has an optimum growth temperature of 32-36 (C $\pm$ 1 C) and cannot grow above 44 C. It has an optimal growth pH of 5-6 and germinates at pH 2-8. It has been reported that *A. oryzae* can grow in corn flour with a water content of about 16%. It usually has a water activity (*aw*) above 0.8 in the media, but it grows below 0.8

You can use kojic acid to treat areas with hyper pigmentation or scarring. If you want to use products containing kojic acid to treat a specific condition or cosmetic appearance, make an appointment. See your dermatologist. They can help you determine the best and safest course of action.

2. REVIEW OF LITERATURE

Since tyrosinase is an important enzyme in the synthesis of melanin by melanogenesis, it is an important and successful target for melanogenesis inhibitors that directly inhibit tyrosinase catabolic activity. Most commercially available cosmetics or skin lightening products are tyrosinase inhibitors.

The fact that inhibitors target tyrosinase is that tyrosinase is produced exclusively by melanocytes, which can inhibit melanogenesis in cells without side effects. Many tyrosinase inhibitors use hydroquinone (HQ) arbutin, kojic acid , L-ascorbic acid 40—, ellagic acid, and tranexamic acid. With some drawbacks The review focuses on the

latest findings of tyrosinase inhibitors directly involved in inhibiting tyrosinase catalyst activity and activity from all sources, including laboratory synthetic methods, natural products, virtual screening and structural molecular docking studies. In the present review, inhibitors are mainly classified into two components, further inhibiting fungal and human tyrosinase inhibitors and their chemical activities .

Inhibitors of fungal tyrosinase:

Tyrosinase, a fungus derived from the fungus *Agaricus bisphosphorus*, is frequently used as an enzyme in vitro to produce skin whitening products that target human tyrosinase. Since mushroom tyrosinase (mTAR) is commercially available, most research has been done with this enzyme.

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For screening compounds, popular whitening agents such as kojic acid, arbutin or hydroquinone were used as a positive control. As a result, in recent years, numerous fungal tyrosinase inhibitors have been identified from natural and synthetic sources. Inhibitory strength was expressed as the IC₅₀ value, which is the concentration of the inhibitor required to inhibit half of the enzyme activity when tested.

The K_i value is a reflection of the ligand-binding relationship with the enzyme. A lower K_i value indicates a higher binding relationship, whereas a higher K_i value indicates a lower binding relationship. The K_i value for non-competitive inhibitors is the same numerical value as IC₅₀ for inhibitors, whereas for competitive inhibitors the IC₅₀ is half the numerical value. For in vitro analysis, B16 cells are commonly used because they are relatively easy to grow in vitro and share the mechanisms of melanogenesis of normal human melanocytes.

Salcons and flavonone inhibitors:

Fruits, vegetables, spices, tea and soy based foods are among the most widely distributed natural ingredients that have an inhibitory effect on tyrosinase along with salmon and various biological functions.⁴- A new series of salcon (1) derivatives of (phenylephrine) was synthesized and their inhibitory effects on the diphenolysis activity of banana tyrosinase were evaluated

Results show that 1a-1j compounds inhibit tyrosinase enzyme and in particular 1e and 1i effectively inhibit IC₅₀ values of 0.133 and 0.134 M, respectively.

Furthermore, the compounds were identified as inhibitors

Descendants of Coumarins:

Coumarins are a large family of benzopyrone compounds. In recent studies, some coumarins have been shown to inhibit fungal tyrosinase, with esculetin and umbelliferone having a strong tyrosinase inhibitory activity of 71.72.

Screening is another alternative strategy to detect new inhibitors other than artificial or natural sources. The screening of mainly clinically approved drugs is increasing for many biological targets. Data related to existing medicine will reduce the time and cost associated with intellectual property rights for developing novel drugs.

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The discoloration effect of kojic acid esters synthesized by the esterification of kojic acid using *Rhizomucor miehei* immobilized lipase was studied in B16F1 melanoma cells. The discoloration effect of kojic acid and kojic acid esters was assessed by the inhibition effect of melanin formation and tyrosinase activity on α -stimulating hormone- (α -MSH-) melanin synthesis in B16F1 melanoma cells.

The cellular tyrosinase inhibitory effect of kojic acid monooleate, and kojic acid monolaurate was found to be similar to kojic acid in non-toxic levels ranging from 1.95 to 62.5 g / mL.

However, kojic acid monopalmitate in the range of 15.63 to 62.5 g / mL provided slightly higher inhibition of melanin formation compared to other inhibitors. Kojic acid and kojic acid esters also show antioxidant activity, which enhances the discoloration effect.

Since kojic acid esters have a less cytotoxic effect than kojic acid, it is recommended that kojic acid esters be used as a safe skin whitening agent and as an alternative to potential pigmentation agents for the treatment of hyper pigmentation.

- Schinus terebinthifolius Raddi extract and linoleic acid from Passiflora edulis synergistically decrease melanin synthesis in B16 cells and reconstituted epidermis.

Synthesis of tyrosinase inhibitory Jorge AT, Arroteia KF, Santos IA, Andres E, Medina SP, Ferrari CR, Lourenço CB, Biaggio RM, Moreira PL. Int J Cosmet Sci. 2012

- kojic acid derivative.

Lee YS, Park JH, Kim MH, Seo SH, Kim HJ. Arch Pharm (Weinheim). 2006 Mar;339(3):111-4. doi: 10.1002/ardp.200500213

- Conjugation with Dihydrolipoic Acid Imparts Caffeic Acid Ester Potent Inhibitory Effect on Dopa Oxidase Activity of Human Tyrosinase.

5

- Evaluation of depigmenting activity by 8-hydroxydaidzein in mouse B16 melanoma cells and human volunteers.

Tai SS, Lin CG, Wu MH, Chang TS. Int J Mol Sci. 2009 Nov 20;10(10):4257-66. doi: 10.3390/ijms10104257. PMID: 20057943 Free PMC article.

- Effect of the combination of different depigmenting agents in vitro.
- Implications of Oxidative Stress in the Pathogenesis and Treatment of Hyperpigmentation Disorders.
- Design, synthesis, and biological evaluation of symmetrical azine derivatives as novel tyrosinase inhibitors.
- Antioxidative and Anti-inflammatory Effects of Kojic Acid in A β -Induced Mouse Model of Alzheimer's Disease.
- Evaluation of 4-(4-Fluorobenzyl)piperazin-1-yl]-Based Compounds as Competitive Tyrosinase Inhibitors Endowed with Antimelanogenic Effects.
- Pigmentation Levels Affect Melanoma Responses to Coriolus versicolor Extract and Play a Crucial Role in Melanoma-Mononuclear Cell Crosstalk.

3. AIM AND SCOPE

3.1 AIM :

- To prepare skin lightening cream from kojic acid extracted from fungi *A.oryzae*

3.2 OBJECTIVES:

- Purchasing culture
- Production of kojic acid using synthetic medium
- Estimation of kojic acid using DCIP method (2,6 Dichlorophenolindophenol)
- Optimization of kojic acid production along with other bioactivities antibacterial ,antioxidant, anti tyrosinase activity produced kojic acid from optimized medium.
- Cream formulation

3.3 PLAN OF WORK

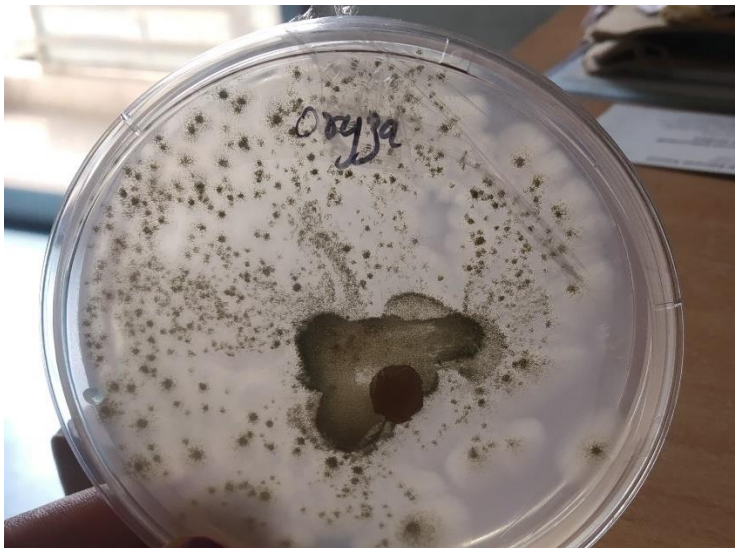
- Preparation of PDA (potato dextrose agar)
- Preparation of synthetic media
- Enaculating of *A.oryzae*
- Selecting the culture
- Using plant samples
- Plant sample preparation
- Enaculating *A.oryzae* in plant samples
- DCPIP estimation
- Percentage of inhibition calculation
- Ethyl acetate solvent extraction
- Antioxidant for ethyl acetate extract.
- Antityrosinase test for ethyl acetate extract
- GCMS analysis
- Preparation of cream
- Kojic acid amended formulation

- To check antioxidant & antityrosinase activity for final product formulation

4.MATERIALS AND METHODOLOGY

4.1 MATERIALS

Sample collection: *A.oryzae* was collected and grown into a media. And for other bioactivities floral extracts such as Rose, orange, hibiscus were Dried and ground into powder.



4.2. MEDIA PREPARATION

4.2.1 Potato Dextrose Agar (PDA)

Potato Dextrose Agar (PDA) is used for the cultivation of fungi. Potato Dextrose Agar (PDA) is a general purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth. It is recommended for plate count methods for foods, dairy products and testing cosmetics.

4.2.2 Composition of Potato Dextrose Agar (PDA)

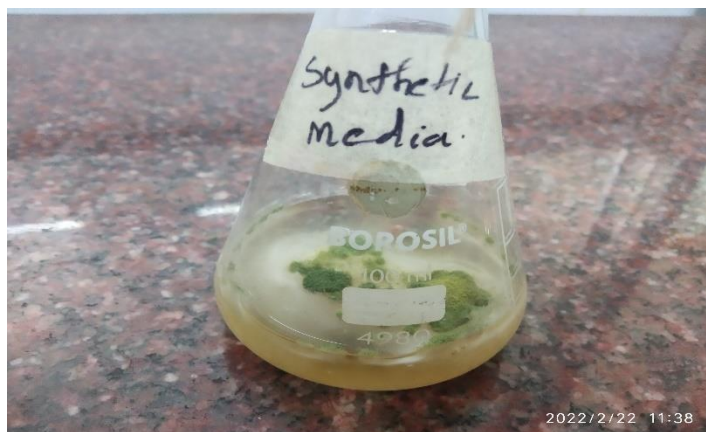
- Potato infusion - 200 gm
- Dextrose - 20 gm
- Agar - 20 gm
- Distilled water - 1 liter

4.2.3 Procedure of Potato Dextrose Agar (PDA)

- To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 liter distilled water for 30 min.
- Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form).
- Mix with Dextrose, Agar and Water and boil to dissolve.
- Autoclave 15 min at 121°C.
- Dispense 20-25 ml portions into sterile 15 × 100 mm petri dishes.
- Final pH, 5.6 ± 0.2 .

4.2.4. Synthetic medium:

Such media are composed of the substances that are chemically known. These media are very useful in studying the physiology, metabolic nature and nutritional requirements of microbes. Both autotrophs and heterotrophs can be grown in these media.



4.2.5. Composition of Synthetic medium:

- Glucose - 50 gm
- Yeast extract - 5gm
- Mg sulphate - 0.5gm
- Potassium dihydrogen phosphate- 1.5gm
- Distilled water - 250 ml

4.2.6. Procedure of Synthetic media

- To prepare synthetic media add the necessary ingredients into the distilled water
- stir the medium to get the powder
- distribute the medium into tubes/flasks
- autoclave to sterilize the media
- autoclave the synthetic medium for plate production and then pour into sterile petri dishes/ cool them for fungal inoculation.

4.2.7 Enaculation of A.oryzae

A. oryzae have the ability to grow on media , potato dextrose agar on which the growth is fast with heavy colonies,

The liquid medium flasks were inoculated with fungal cultures A.oryzae, and the culture is placed in a shaker for 7-10 days to see the growth of fungi.

4.2.8. Selecting the culture.

After 5-7 days the A. oryzae has an optimal growth temperature of 32–36 °C (± 1 °C) and is unable to grow above 44 °C. It has an optimal growth pH of 5–6 and can germinate at pH 2–8. It generally can grow on media with a water activity (a_w) above

0.8, but it rarely grows below 0.8. *A. oryzae* extend at the apical tips and multiply by branching, so that the colony covers the surface of the solidified agar medium after several days of incubation. When grown on the surface of an agar medium, the colony is initially white because of the vegetative hyphal growth, and then it turns to yellowish green as a large amount of conidia form. In most strains of *A. oryzae*, the color of fresh culture or conidia is yellowish green, but that of old culture is brown, sometimes green with brown shades.



4.2.9. Adding plant samples :

Plants were once the main source and foundation of all cosmetics before the discovery of methods of combining materials with similar properties. The use of plant extracts in cosmetics is increasing, largely due to the poor image that animal extracts have received over the past few years. In part, this is due to news reports related to bovine spongiform encephalitis (BSE). Materials derived from certain animals need to be replaced, but synthetic chemicals cannot always do this. Natural molecules derived from plant extracts provide a particularly exciting way for further research.

Plant extracts differ from purified therapeutic agents in many respects. First, they are more dilute than the pure chemicals we are familiar with; Second, herbs often have

additional active principles that are closely related chemically and therapeutically to the components primarily responsible for its effects. , The application of some select extract was introduced in various areas: skin irritation licorice; Rose extract helps to cleanse the skin and prevent blemishes; Orange extract helps against aging, plants with anti-inflammatory properties often contain high levels of flavonoids; Used to strengthen and tone the skin, it is rich in tannins, which have an astringent effect; And the use of plants containing many antibiotics and antifungal antibiotics to heal the skin during infections is beneficial

So the selected plant samples are Rose, Orange, Hibiscus



4.3 ROSE

As a mild antiseptic and abrasive, it helps to cleanse the skin and prevent blemishes. The anti-inflammatory properties of rose juice help to reduce redness and soothe irritation - it is excellent for all skin types (acne, dry, mature, sensitive) due to its calming and healing properties. It evokes skin whitening and anti-wrinkle formation activity by regulating intracellular signaling, supporting its utility as an ingredient for skin whitening and anti-wrinkle cosmetic products.

4.3.1 Orange:

Orange is one such fruit that plays a significant role when it comes to skin whitening. Due to its exfoliating activity, Orange Extract is recommendable to formulate a high range of cosmetic products such as antiaging, moisturizing or depigmenting cosmetic products and cosmetic products aimed at improving general blood circulation. Orange extract has a gentle astringent property that helps to relieve acne-prone skin. It's gentle and reduces itching and irritation ./while also reducing the acne and preventing scarring. Orange oil can also correct the oil balance of the skin.

4.3.2 Hibiscus:

The natural surfactants property of hibiscus helps in deep cleansing the skin without depriving of its natural oils. The AHAs in hibiscus remove dead cells making the skin look fresh. The chock full of antioxidants, anthocyanocides in hibiscus play an amazing role in improving skin health by guarding it from radical damage and delaying the premature ageing of the skin. The astringent property of hibiscus controls the excess secretion of oil by tightening the pores while its anti-inflammatory properties of hibiscus lessens inflammation and heal redness of the skin. For any uneven skin tone, hyperpigmentation, dark spots and discolouration, hibiscus works as an instant remedy.

4.3.3 MEDIA PREPARATION /PLANT SAMPLE PREPARATION

Synthetic media broth was prepared for 50 ml for three plant samples (rose, orange, hibiscus), and 5gm of each plant extract is added to it , and the sterilization process is carried out, the *A.oryzae* is enaculated in the fresh culture. afte5 3-5 Days the growth of *A.oryzae* is seen in those plant samples. the fungal growth is found to be more in the rose plant sample.



4.3.4 Quantitative Determination of Kojic Acid

Kojic acid was determined using a spectrophotometric method with 2,6 dichlorophenolindophenol (DCPIP)

It is often necessary to determine exactly the kojic acid which is produced in the fermentation medium

2,6-dichlorophenolindophenol, (DCPIP) is a blue chemical compound used as a redox dye. Oxidized DCPIP is blue, reduced DCPIP is colorless.

The rate of photosynthesis can be measured by the rate at which this dye is broken down (reduced) when exposed to light in a photosynthetic system. This reaction is reversible, as colorless DCPIP can be reoxidized to blue. It is often used in measurements of the electron transport chain in plants because of its higher [affinity] for electrons than ferredoxin. For example, it is possible to compare rates of photosynthesis with DCPIP. Due to the reductions that occur during the light

reactions, DCPIP can be substituted for NADPH, the final electron carrier in the light reactions. The light reactions will reduce the DPIP and turn it colorless. As DCPIP is reduced and becomes colorless, the following increase in light transmittance can be measured by a spectrophotometer.

4.3.5 Reduction of DCPIP:

DCPIP is a redox dye commonly used as a monitor of the light reactions in photosynthesis because it is an electron acceptor that is blue when oxidized and colourless when reduced. DPIP is commonly used as a substitute for NADP⁺. The dye changes color when it is reduced, due to its chemical structure. The nitrogen atom in the center of the molecule is the atom that accepts electrons, and it changes the double N-C bond to a single bond, which forces bonds between carbons in the entire left ring to change. This microscopic shift in the DCPIP structure causes the macroscopic change in colour, from dark blue to colourless.

The rate of photosynthesis light-dependent reaction can be measured with this property of DCPIP, because one of the stages of the light reaction is an electron transport chain that normally ends with the reduction of NADP⁺. When DCPIP is present, it also gets reduced by the light reaction. The amount of DCPIP reduced can be found by measuring the solution's light transmittance with a spectrophotometer.



4.3.6. Percentage of inhibition :

% of Inhibition	ROSE	ORANGE	HIBISCUS
C1	0.024	0.0071	0.094
C2	0.027	0.067	0.093
C3	0.017	0.067	0.060
C4	0.015	0.071	0.067
C5	0.006	0.076	0.071g
C6	0.095	0.095	0.095

$$\% \text{ of inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

4.3.7. Percentage of Inhibition calculation:

% of Inhibition	<u>ROSE</u>	<u>ORANGE</u>	<u>HIBISCUS</u>
C1	74.7	25.2	10.5
C2	71.6	29.4	2.10

C3	82.1	29.4	36.8
C4	84.2	25.2	29.4
C5	93.6	20.2	25.2

4.3.8. Antityrosinase activity test:

Melanin is a pigment that occurs in humans, fungi, and plants. It is responsible for the colour of eyes, hair, and skin in humans . The pigment is secreted and produced by the melanocytes cells, which are distributed in the basal layer of the dermis, through a physiological process called melanogenesis . It is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of the enzyme tyrosinase. The key enzyme that is responsible for melanin production is tyrosinase . Hyperpigmentation of the skin occurs due to overactivity of tyrosinase enzyme. The aim of this study was to test the effect of the selected plant extracts on tyrosinase enzyme and to identify which plant extracts can be used as possible skin-lightening agents. Tyrosinase, also known as polyphenol oxidase, is a copper containing monooxygenase that catalyzes two distinct reactions involving molecular oxygen

4.3.9 Composition of Tyrosinase Enzyme Assay:

The assay was performed using

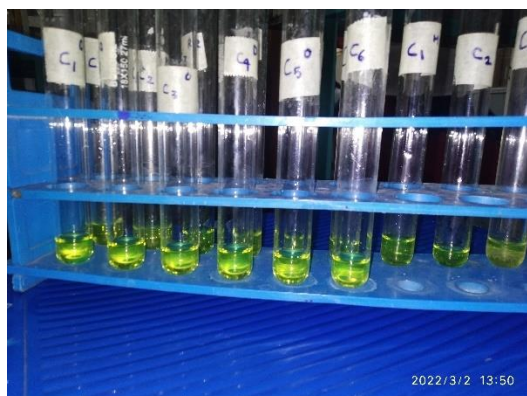
Phosphate buffer -1.9ml

Mushroom tyrosinase extract-100 μ l

Plant sample -100 μ l

L-dopamine -1.5 milli molar/.w;

Incubate for 30 mins , And read at 475 nm



% of Inhibition	ROSE	ORANGE	HIBISCUS
0	0.623	0.622	0.672
1	0.562	0.564	0.763
2	0.493	0.551	1.006
3	0.665	0.660	1.126
4	0.628	0.598	0.695
5	0.702	0.682	0.701

$$\% \text{ of inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

% of Inhibition	ROSE	ORANGE	HIBISCUS
1	9.791	9.324	-13.541
2	20.86	11.414	-49.702
3	-6.741	-6.109	-6.7559
4	-0.8025	3.858	-3.422

4.4 ANTIOXIDANT SOD ASSAY

Superoxide anion radical scavenging activity

Superoxide radicals were generated by method described by Beauchamp and Fridovich (1971) with slight modification. Superoxide radicals are generated in riboflavin, methionine, illuminate and assayed by the reduction of NBT to form blue formazan (NBT²⁺). All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The different concentration of rice extract was added in the reaction mixture. The total volume of the reactant mixture was 3 mL and the concentrations of the riboflavin, methionine and NBT were 1.33×10^{-5} , 4.46×10^{-5} and 8.15×10^{-8} M, respectively. The reactant was illuminated at 25°C for 40 min. The photochemically reduced riboflavins generated superoxide which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. The reaction mix not containing sample was served as control. The absorbance was measured at 560 nm. Rice extract was added to the reaction mixture, in which superoxide was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity.

The inhibition percentage of superoxide anion generation was calculated by using the following formula:

Superoxide radical scavenging activity =

$$\{(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})\} \times 100$$

Where; Abs control is the absorbance of illuminated superoxide radical; Abs sample is the absorbance of superoxide radical + sample extract.

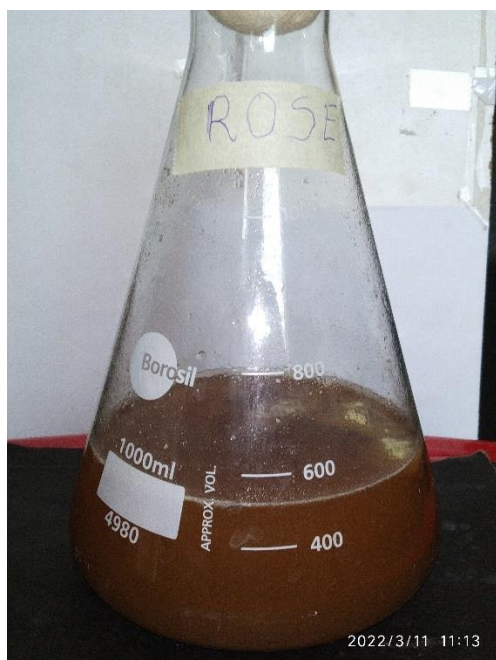
% of Inhibition	ROSE	ORANGE	HIBISCUS
C1	0.024	0.071	0.885

C2	0.027	0.067	0.569
C3	0.017	0.067	0.060
C4	0.015	0.071	0.067
C5	0.006	0.076	0.067
C6	0.015	0.015	0.015

4.5. **Ethyl acetate solvent extraction:**

Solvents like ethyl acetate are used in extraction processes because of its chemical and biological functions such as medium polarity and minimum cell toxicity. Ethyl acetate biphasic actions enables it to be used to extract both polar and non polar compounds.

The product should be washed with water, extracted using ethyl acetate and then purified by silica gel column chromatography. The product is not a precipitate. It is liquid, soluble in both water and ethyl acetate. When I add water to the product and then add ethyl acetate to it, two layers are formed. Very little compound comes into ethyl acetate.



4.5.1 **Procedure:**

Obtain a separatory funnel

- If the separatory funnel has a Teflon stopcock, reassemble the stopcock if it was taken apart to dry, placing the parts in the appropriate order . Be sure that the Teflon stopcock is moderately tight so that it can still easily turn, but is not so loose that liquid can seep around the joint.
- If using a glass stopcock it likely needs no further preparation. There should be a very thin layer of grease used to seal the stopcock and prevent freezing. If both glass and Teflon stopcocks are available, Teflon is a better choice as there is always a possibility that solvent can dissolve the grease used with glass stopcocks and contaminate the sample.

Also obtain a stopper (Teflon or ground glass) that fits well in the top joint of the funnel

- Place the separatory funnel in a ring clamp attached to a ring stand or latticework. The funnels are easy to break, so cushion the funnel in the metal clamp using pieces of slit rubber or plastic tubing

4.5.2 Add the Solutions (for single extraction)

- Before pouring anything into a separatory funnel, be sure that the stopcock is in the "closed" position, where the stopcock is horizontal

As a fail-safe, always position an Erlenmeyer flask beneath the separatory funnel before pouring This can catch liquid in case the stopcock is accidentally left open, or if the stopcock is loose and liquid leaks through unintentionally.

- Using a funnel, pour the liquid to be extracted into the separatory funnel A separatory funnel should never be used with a hot or warm liquid.

The ground glass joint atop a separatory funnel is more prone to stick to the stopper if there was liquid in the joint at some point. Pouring liquid into the separatory funnel using a short-stemmed funnel avoids getting the joint wet, so that it will be less likely to freeze during mixing.

- Pour a quantity of the extractive solvent into the separatory funnel, as indicated by the procedure .

It is unnecessary to use precise quantities of solvent for extractions, and the volumes can be measured in a graduated cylinder. If a procedure calls for 20mL20mL of solvent, it is acceptable if between 2020-25mL25mL is used each time.

4.5.3. Mix the Solutions (for single extraction)

- Place the stopper on the funnel, and hold the funnel such that the fingers of one hand securely cover the stopper, while the other hand grips the bottom of the funnel
- Gently invert the funnel, and swirl the mixture a little.

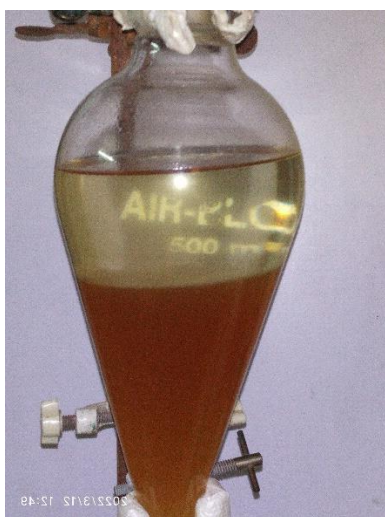
Although it is not uncommon for *some* liquid to creep into the ground glass joint when inverted, it should be minimal. If liquid drips onto your fingers or gloves when you invert the funnel, the stopper is probably the wrong size.

- Pressure may build up inside the separatory funnel when solutions are mixed, so immediately after swirling, and with the funnel still inverted, "**vent**" the funnel by briefly opening the stopcock to allow for a release of pressure .

Pressure builds in the funnel as solvent evaporates into the headspace and contributes additional vapor to the initial ~ 1 atmosphere of air pressure in the funnel. With highly volatile solvents (like diethyl ether), a definite "swoosh" can be heard upon venting, and small amounts of liquid may even sputter out the stopcock. If liquid spits out the stopcock, try to allow it to drain back into the funnel. The noise associated with venting normally ceases after the second or third inversions, as the headspace becomes saturated with solvent vapors and the pressures inside and outside the funnel are equalized.

- Close the stopcock and mix the solutions a bit more vigorously, periodically stopping to vent the system.

- There are differences of opinion on how vigorously solutions should be mixed in separatory funnels, and for how long. As a general guide, a mild mixing for **10-20 seconds** should be enough. With some solutions (e.g. dichloromethane), care should be taken to not shake too vigorously, as these solutions often form emulsions (where the interface between the solutions doesn't clarify). With solutions prone to emulsions, a funnel should be gently *rocked* for one minute.
- Place the separatory funnel upright in the ring clamp to allow the layers to fully separate. The interface between the layers should settle rather quickly, often within 10 seconds or so. If the interface is clouded or not well defined (an emulsion has formed), see the troubleshooting section for tips.



4.5.4 Separate the Layers (for single extraction)

- Liquid will not drain well from a separatory funnel if the stopper remains on, as air cannot enter the funnel to replace the displaced liquid. If liquid did drain from the funnel without replacement by an equal volume of air, a negative pressure would form in the funnel. Thus, before draining liquid from a separatory funnel, remove the stopper .
- Drain the majority of the bottom layer into a clean Erlenmeyer flask, positioning the ring clamp so that the tip of the separatory funnel is nestled in the Erlenmeyer flask to prevent splashing , Stop draining when the interface is within 1cm of the bottom of the stopcock.

- Gently swirl the funnel to dislodge any droplets clinging to the glass ,A glass stirring rod can be used to knock down stubborn clinging droplets.
- Further drain the bottom layer, stopping when the interface just enters the stopcock chamber Label the Erlenmeyer flask (e.g. "bottom layer").
- When labeling flasks, it's often best to use terminology that is without question correct, such as "top layer" or "bottom layer". If the layers are labeled with statements like "organic layer" or "aqueous layer", it's possible that the layers have been incorrectly identified. It may be best to use combined statements like "top organic layer" as it's quite useful to track the aqueous and organic layers. If the layers have been incorrectly identified, at least the "top" part of the label will always be correct.
- Pour out the top layer from the top of the separatory funnel into another clean Erlenmeyer flask making sure to again label this flask.
It is proper technique to drain the bottom layer through the stopcock, and to pour out the top layer from the top of the funnel. This method minimizes re-mixing the solutions, as only the lower layer touches the stem of the funnel.

4.6. Cream formulation

Creams are semi solid emulsions which. face cream is essential for every skin type and for every age . Face cream also nourish the skin and keep it healthy – looking . With the help of additives such as emulsifying agent and newer techniques and ingredients, the preparation of creams has become easy

4.6.1 MATERIALS AND METHODS

Ingredients Quantity for 50 g (%)

Oil Phase

- Stearic acid 4.0 %
- Liquid paraffin 8.0 %
- Lanoline 1.0 %
- Glyceryl monostearate 3.0 %

Water phase

- Glycerin 4.0 %
- Propylene glycol 4.0 %
- Isopropyl myristate 2.0 %
- Triethanolamine 0.2 %
- Methyl paraben 0.03 %
- Propyl paraben 0.07 %
- Perfume & Distilled Water to make 50 %

4.6.2 Methodology

Cream formulation based on triethanolamine using stearic acid and emulsifying wax alone and in combination of both. Oil in water emulsion cream was prepared by initially melting wax at 70-80 °C and to the molten mass added stearic acid, liquid paraffin, lanoline, glyceryl monostearate. Aqueous phase along with propylene glycol, isopropyl myristate, triethanolamine, glycerin and water heated at same temperature as oil phase.

Both the phases were mixed slowly with continuous stirring to form the homogenous dispersion. Perfume was added when the temperature downs at 35 °C.

Preservatives added in the water phase before mixing

4.6.3 Kojic acid amended formulation

The kojic acid infused plant sample which was collected after ethyl acetate solvent extraction is now mixed and blended into this prepared cream.



RESULTS AND DISCUSSION

5. Antityrosinase activity for ethyl acetate extract.

In order to treat hyperpigmentation-related problems, there has been a global trend in developing cosmetics claiming to have skin-whitening properties, which act by inhibiting melanin biosynthesis. This can be achieved by protecting the skin from solar light exposure, removing melanin by corneal ablation, blocking or diminishing the proliferation of melanocytes or inhibiting tyrosinase activity, since melanin is derived from tyrosine and its biosynthesis is to a large extent regulated by this copper-containing enzyme.

5.1 Composition of Tyrosinase Enzyme Assay:

The assay was performed using

Phosphate buffer -2ml

Mushroom tyrosinase extract-100 μ l

Tyrosinase enzyme-100 μ l

L-dopamine -10 μ l;

Sample- 50 μ l

(In 100 ml of rose extract (after ethyl acetate extraction) mix 20 mg of cream. And together mix them in 100 μ l distilled water)

Heat killed – 0.286

Control - 0.186

Phosphate buffer – 0.150

5.1.2 Effective concentration of tyrosinase activity

- Control 20 mg cream + 10 μ l Distilled water
- Blank – Distilled water
- In Sample 1 add 20 mg cream and 10 μ l extract
- In sample 2 add 20 mg cream and 20 μ l extract
- In sample 3 add 20 mg cream and 50 μ l extract
- In sample 4 add 20 mg cream and 100 μ l extract
- In sample 5 add 20 mg cream and 200 μ l extract
- Incubate for 20mins and read at 475 nm

Control	OD value
1	36.94
2	86.50
3	78.91
4	66.84
5	85.35

5.2. GC MS Analysis

Methodology Adopted:

Gas Chromatograph: A Shimadzu GC-2010 Plus gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250 μ I.D., 0.25 μ film thickness). A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35°C, hold for 2 minutes, then ramp at 20°C per minute to 450°C and hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode). The sample was dried under nitrogen and derivatized with 300 μ l of trimethyl silane at room temperature for 20 min. The derivatized sample was dissolved in 1 ml of toluene. A volume of 1 μ l was injected at the time of analysis.

Mass Spectrum: A Direct connection with capillary column metal quadrupole mass filter pre rod mass spectrometer operating in electron ionization (EI) mode with software GCMS solution ver. 2.6 was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 1000 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 1000 at 1 second per scan

PEAK	NAME	R.TIME	AREA	AREA%	HEIGHT %
1	2-FURANMETHANOL	3.531	9528556	0.91	1.22

2	PROPANOIC ACID, 2- HYDROXY-, E	3.626	10164881	0.97	0.87
3	Oxime-, methoxy- phenyl-	3.757	1829143	0.17	0.63
4	Benzoic acid, 2-methoxy-, methyl ester	3.797	8323474	0.79	1.11
5	1,2- PROPANEDI OL	4.033	27948382	2.66	2.39
6	Oxime-, methoxy- phenyl-	4.141	23650176	2.25	2.35
7	Isopentyl propyl carbonate	4.295	11375196	1.08	1.37
8	.ALPHA.,,BE TA.- CROTONOL ACTON	4.375	5423235	0.52	1.14
9	2- CYCLOHEX EN-1-OL	4.434	10432108	0.99	1.36
10	1,2- CYCLOOCT ANEDIONE	4.540	16747836	1.60	2.06
11	1,3- Propanediol, trimethylsilyl ether	4.667	5291586	0.50	0.57
12	Cyclopropane carboxamide, N-n-butyl	5.624	4507125	0.43	0.49
13	ACETIC ACID, PHENYL ESTER	5.721	3377139	0.32	0.53
14	2-Hydroxy- gamma- butyrolactone	5.831	10849618	1.03	0.78
15	1,2,3- PROPANET RIOL	5.931	3877651	0.37	0.81
16	1,2,3- PROPANET RIOL	5.972	4030137	0.38	0.83
17	2- FURANCAR BOXYLIC ACID, TETR	6.027	6490066	0.62	0.84
18	1,2,3- PROPANET RIOL	6.090	10920676	1.04	0.79

19	CYCLOPROPYLMETHANOL	8.104	20177673	1.92	2.45
20	Ethyl .alpha.-D-glucopyranoside	21.171	8790993	0.84	0.51
21	Silane, [3-(2,3-epoxypropoxy)propyl]tetra-	21.622	2982097	0.28	0.38
22	Hexadecanoic acid, methyl ester	26.518	2825610	0.27	0.55
23	9,12-OCTADECADIENOIC ACID (Z,Z)	28.681	2188858	0.21	0.51
24	9-OCTADECENOIC ACID, METHYL	28.752	5402718	0.52	0.76
25	<NO NAME>	31.525	2295013	0.22	0.27
26	<NO NAME>	31.605	4125733	0.39	0.31
	R.Time	Area	Area%	Height%	
	32.271	177164911	16.89	10.41	
28	Stigmasta-5,24(28)-dien-3-ol, (3.beta.)-	32.301	48042595	4.58	11.11
29	Fucosterol	32.446	335175485	31.95	16.48
30	LUP-20(29)-ENE-3,28-DIOL, (3.BETA	32.956	5143311	0.49	0.86
31	LUP-20(29)-ENE-3,28-DIOL, (3.BETA	33.000	9296817	0.89	0.93
32	HAHNFETT	33.142	1982457	0.19	0.26
33	Gorgost-5-en-3-ol, (3.beta.)-	33.592	4395092	0.42	0.57
34	STIGMAST-5-EN-3-OL, (3.BETA.,24	33.631	5622486	0.54	0.55
35	Sitosterol	34.197	8026022	0.77	0.77
36	Humulane-1,6-dien-3-ol	35.824	4963088	0.47	0.52
37	Squalene	36.139	3748131	0.36	0.94
38	Stigmasta-4,24(28)-	36.300	30216090	2.88	1.96

39	dien-3-one, (24E)- Bicyclo[4.4.0]]dec-6-en- 9.beta.-ol, 1,7-d	36.537	1890489	0.18	0.26
40	Isocarpesterol	36.638	6223843	0.59	0.64
41	9,19- CYCLOLAN OSTAN-3- OL, 24-M	36.805	10723037	1.02	0.75
42	26,27- DINORERG OSTA-5,23- DIEN-3	37.407	47266171	4.51	9.76
43	Ergosta-5,22- dien-3-ol, (3.beta.,22E)-	37.451	90206898	8.60	11.90
44	22,23- Dibromostig masterol acetate	37.610	20168638	1.92	2.10
45	PREGN-7- ENE, (5.ALPHA.)-	37.695	3260363	0.31	1.14
46	26,27- Dinorergosta- 5,23-dien-3- ol, (3.b	37.743	5528725	0.53	1.02
47	Humulane- 1,6-dien-3-ol 100.00	37.949	6410862 100.00	0.61	1.18

5.3. Cytotoxicity analysis

MATERIALS AND METHODS

13.1.Preparation of cell suspension

A subculture of HacaT cells in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized separately, after discarding the culture medium. To the

disaggregated cells in the flask, 25 mL of DMEM with 10% FCS was added. The cells were suspended in the medium by a gentle passage with the pipette and the cells homogenized.

13.1.2 Seeding of cells

One mL of the homogenized cell suspension was added to each well of a 96-well culture plate along with the different concentrations of sample Kojic acid formulation (0 to 200 µg/mL) and incubated at 37°C in a humidified CO₂ incubator with 5% CO₂. After 48 hrs incubation, the cells were observed under an inverted tissue culture microscope. With 80% confluence of cells, a cytotoxicity assay was carried out.

13.1.3 Cytotoxicity assay

The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial Succinate dehydrogenase and reductase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. After 48 h incubation, the wells were added with MTT and left for 3 h at room temperature. All wells have removed the content using a pipette and 100µl SDS in DMSO was added to dissolve the formazan crystals, absorbances were read in Read Well Touch microplate reader at 570 nm (Mosman *et al* 1983).

5.4 RESULTS

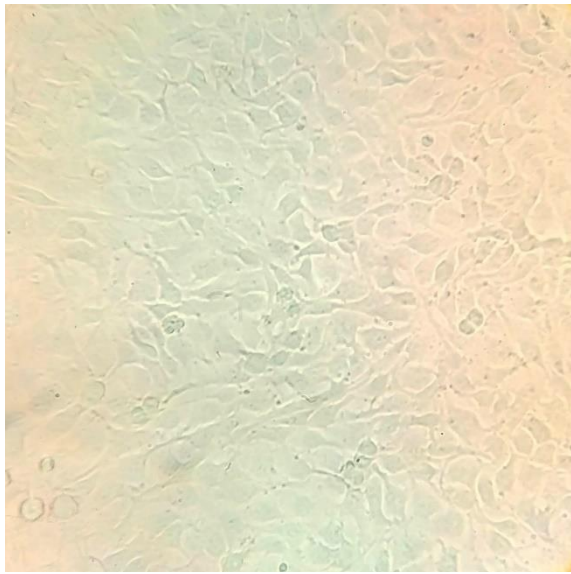
The in-vitro cytotoxicity activity studies were shown that HacaT cell lines were not affected by the different concentrations of test samples (Table 1). In the HacaT cell line .cytotoxicity effect was not observed in the tested sample in all the concentrations at 48 hours of treatment, it also revealed that increased concentration of test samples showed slightly increased cytotoxicity over the HacaT cell line

(Figure 1). The IC₅₀ concentration of the given sample was 1230.44 (µg/ml) against the HacaT Cell line (Figure 2).

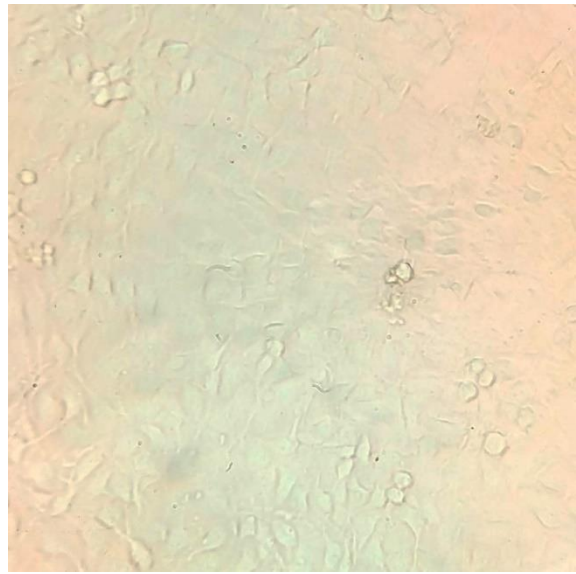
Table 1: In vitro cytotoxicity effect of Kojic acid formulation against HacaT cell line

Sample Concentrations (µg/ml) HacaT Cell Viability (%)

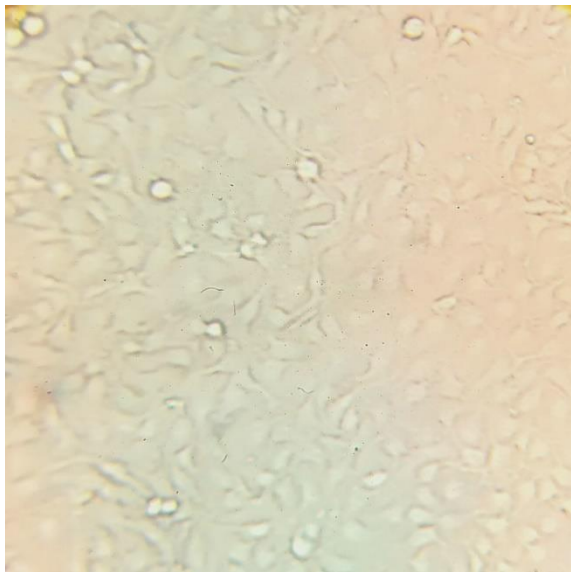
Sample Concentrations (µg/ml)	HacaT Cell Viability (%)
0.0	100.00
6.3	98.54
12.5	96.91
25.0	95.56
50.0	94.72
100.0	92.59
200.0	91.18



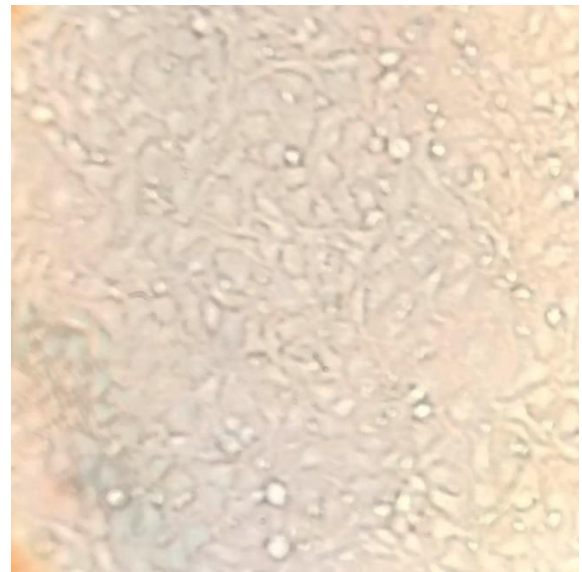
Control



50 µg/mL



100 µg/mL



200 µg/mL

Figure 1: Cytotoxicity activity of Kojic acid formulation against HacaT cell lines

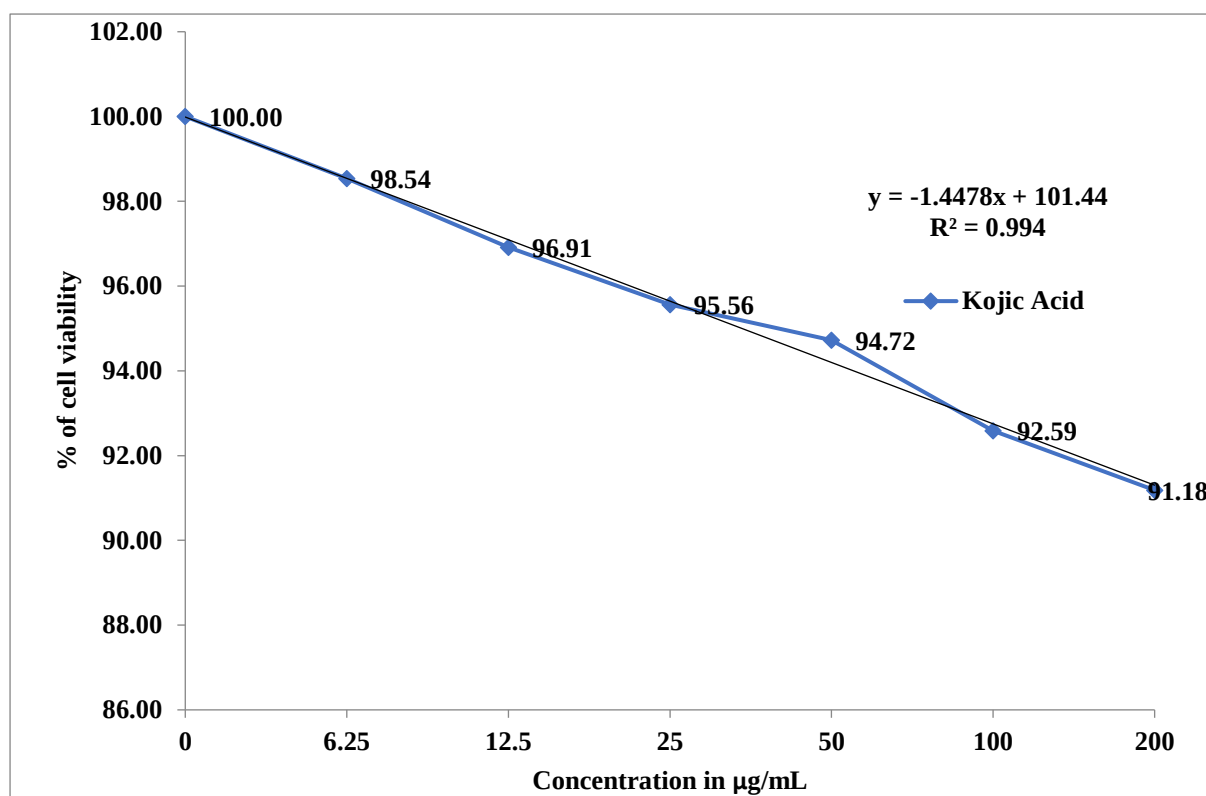


Figure 2: Cytotoxicity activity of Kojic acid formulation against HacaT cell lines after 48h

6. SUMMARY AND CONCLUSION

My purpose in this project is to formulate a kojic acid cream to reduce the hyperpigmentation and lighten the skin tone, along with other bioactivities using floral extracts like rose, orange, hibiscus, The application of Rose extract helps to cleanse the skin and prevent blemishes; Orange extract helps against aging, plants with anti-inflammatory properties often contain high levels of flavonoids; Used to strengthen and tone the skin, it is rich in tannins, which have an astringent effect; And the use of plants containing many antibiotics and antifungal antibiotics along with kojic acid enhance your skin complexion and to heal the skin .

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