Pharmaceutical Sciences 2024: Navigating the Future of Drug Discovery and Development
November 2024

DEVELOPMENT AND EVALUATION OF NATURAL SKIN WHITENING FOMULATION USING CLOVE EXTRACT

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Abstract- The aim of this study was to investigate the antioxidant and antityrosinase properties of clove extract and its components, and to assess the correlation between their antioxidant and antityrosinase activities. The standard solution showed an inhibition of 84.21% in the DPPH method at a concentration of 500µg/mL, whereas the test solution exhibited a 61% inhibition at the same concentration. The methanolic extract of clove was also assessed for antioxidant activity using ferric reducing antioxidant power. The control exhibited a percentage inhibition of 78.28% at a concentration of 500µg/mL, while the test solution showed 25.47% inhibition at the same concentration. The methanolic extract of clove displayed antityrosinase properties. The standard showed an antityrosinase activity result of 86.28% at a concentration of 100µg/mL, while the test sample showed a result of 52.65% at the same concentration. The current study sought to create a natural cream that nourishes, moisturizes, and treats different skin issues. The developed product underwent testing for various attributes including color, appearance, viscosity, acidity, durability assessments, and consumer satisfaction.

Keywords: Melanocyte, Melanogenesis, keratinocytes, antioxidant, Herbal Cosmetic.

Introduction - The amount of Asian women desiring lighter skin tones has significantly risen in the past few years. This is partly due to the fact that many powerful skin-lightening substances have been found, especially those derived from plants. Skin color is mostly determined by the level of melanin, a pigment found in the epidermis. It is secreted by melanocyte cells located in the basal layer of the epidermis. (1) Excessive melanin production can result from reactive oxygen species (free radicals), melasma, prolonged sun exposure, and other hyperpigmentation disorders. Excessive melanin production is not preferred as it could lead to an uneven or darker complexion. Changes in color are also desired for aesthetic reasons. The initial stages of melanin production, known as melanogenesis, are triggered by the enzyme activity of tyrosinase.

Herbal products assert that they do not have the usual negative impacts associated with artificial ingredients. The popularity of herbal remedies has resulted in an excessive supply in the Indian market due to advancements in technology and social factors. Hence, we aimed to select plants known for their anti-tyrosinase properties to create a botanical cream. Plants used in cosmetics have multiple properties like anti-tyrosinase, antioxidant, anti-inflammatory, antiseptic, and antibacterial properties. Please provide the text you would like me to paraphrase.

The aromatic dried flower buds of the clove tree (Syzygium aromaticum) are part of the Myrtaceae plant family. Clove essential oil is used for its qualities of being anti-inflammatory, anti-mutagenic, and antioxidant (5). The main goals of this study were to examine the antioxidative and tyrosinase inhibiting properties of clove oil and its components in vitro, as well as their effects on dermal fibroblasts. (6, 7, 8)

THE BIOSYNTHESIS OF MELANIN

Gertrude (2000) stated that Melanin biosynthesis is a multipart pathway that found in highly specialized cells, called melanocytes, within membrane-bound organelles referred to as melanosomes. Melanosomes are transferred by dendrites to surrounding keratinocytes, where they play a critical role in photo protection. The anatomical relationship between keratinocytes and melanocytes is known as “the epidermal melanin unit” and it has been estimated that each melanocyte is in contact with 40 keratinocytes in the basal and supra basal layers. Several important steps must occur for the proper synthesis and distribution of melanin, as follows. (5, 6)

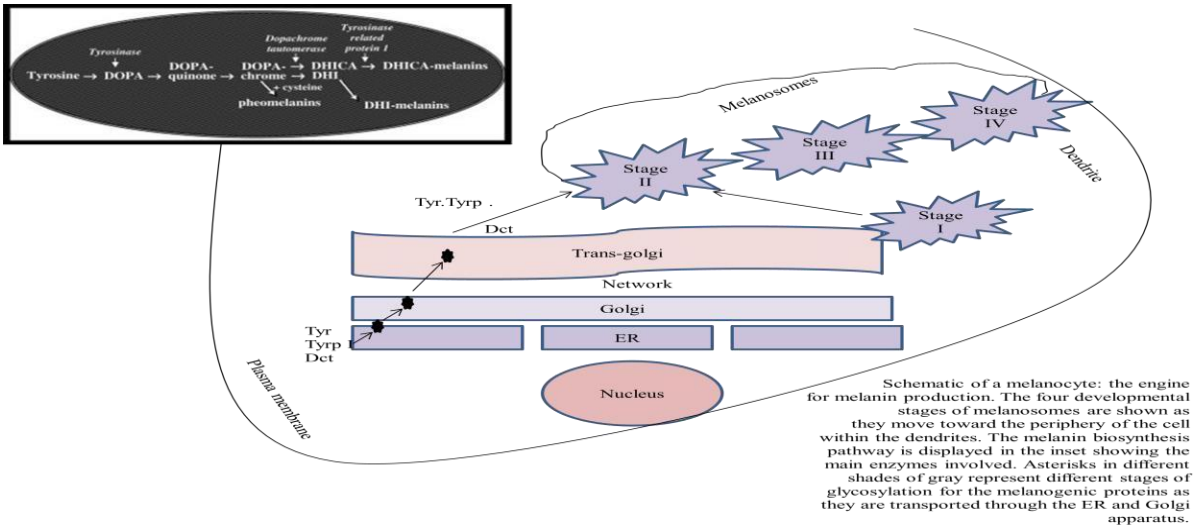


Figure 1.1 – Bio-synthesis of Melanin

Materials and methods:

Clove buds were collected from local market of Meerut and the plant material authenticated by the Natural Bureau of plant genetic resource (NBPGR), Delhi.

Extraction process of Syzygium aromaticum (clove)-

The clove buds was weight (15gm) and powered with mortar pestle. Powder was transferred into conical flasks and extracted with 90 ml of methanol. The conical flask was kept over rotatory shaker on 240-340 rpm for two days at room temperature. The extract was filtered and concentrated by rotatory evaporator. The concentrated solution was collected and stored in deep freezer until further use.

EVALUATION PARAMETERS OF METHANOLIC EXTRACT OF CLOVE

The naturally obtain extract was evaluated for its antioxidant and anti-tyrosinase activity.

a) Antioxidant activity

The methanolic extract of clove has antioxidant activity; this activity was determined using two different types of method.

1. DPPH Radical Scavenging Activity

The DPPH free radical method was use for antioxidant activity of extracts. The lower absorbance of the reaction mixture was indicating higher free radical scavenging activity.

Preparation of solutions for DPPH method

Preparation of standard solution-100mg of Ascorbic acid was dissolved in 100 mLof methanol and drawn 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 mL of this solution volume make up

10mL to give the concentration of 50, 100, 150, 200, 250, 300, 350 and 400µg/mL.

Preparation of test sample-Stock solutions of samples were prepared by dissolving 20 mL of methanolic extract in 20 mL of methanol and drawn 0.5, 1, 1.5, 2, 2.5.3, 3.5 and 4 mL of this

solution volume make up 10mL to give the concentration of 50, 100, 150, 200, 250, 300, 350 and s400µg/mL.

Preparation of DPPH solution-10mg of DPPH was dissolved in 10 mL methanol: it was protected from light by covering the test tubes with aluminum foil.1mL was drawn and again dissolved up to 100 mL of solvent to give 10µg / mL of DPPH solution.

Protocol for DPPH free radical scavenging activity

- 10µg/mL solution of DPPH, absorbance was taken immediately at 516 nm for control reading.
- Different dilutions of test and standard solution were taken into volumetric flask and 1ml of each concentration taken in to volumetric flasks.
- Same volume of DPPH solution was added to each test tube.
- Absorbance was taken at 516 nm in UV spectrophotometer (on visual UV spectroscopy Pharmaspec-1700, Shimadzu, Japan). Methanol used as a blank.

The percentage reducing power of DPPH free radical method was calculated by using equation no.1-

$$\% \text{ Reducing power} = \frac{\text{Control absorbance - Sample absorbance} \times 100}{\text{Control absorbance}}$$

2. Ferric Reducing antioxidant power

The ferric reducing antioxidant power of extract was determined according to the method of Oyaizu (1986).

Preparation of solutions for ferric reducing antioxidant power method

Preparation of standard solution- 100mg of ascorbic acid dissolved in 100 mL of distilled water. Dilutions of this solution with 10mL distilled water were prepared to give the concentration of 50, 100, 200, 300, 400 and 500µg/mL.

Preparation of test sample- 20mL of methanolic extract was dissolve in 20 mL methanol. Dilutions of this solution with 10mL methanol were prepared to give the concentration of 50, 100, 200, 300, 400 and 500µg/ml.

Preparation of reagents

- Phosphate buffer: 0.2M phosphate buffer of pH 6.8 was prepared according to I.P.

- 1% Potassium ferricyanide solution: 1 gm of potassium ferricyanide was dissolved in 100mL of distilled water.
- 10% Trichloro acetic acid: 10 gm of Trichloro acetic acid was dissolved in 100mL of distilled water.
- 0.1% ferric chloride solution: 0.1 gm of ferric chloride was dissolved in 100mL of distilled water.

Protocol of ferric reducing antioxidant power method-

- Ascorbic acid was used as standard and used different concentration (50,100,200,300,400 and 500µg/mL) of standard and test solution.
- To each of the volumetric flask 2.5mL of 0.2M phosphate buffer (pH 6.8) and 2.5mL of potassium ferricyanide was added.
- The mixture was incubated for 20min at 50C in oven.
- To incubate solutions 2.5 mL of 10% w/v trichloroacetic acid was added and centrifuged for 10min at 3000rpm, 2.5 mL of the solution was taken without disturbing.
- In above 2.5mL solution added 2.5mL of distilled water and 0.5mL of 1% freshly prepared ferric chloride solution was added.
- Absorbance was recorded at 697nm.

The percentage reducing power of ferric reducing antioxidant power method was calculated by using equation no.2-

$$\%\ reducing\ power = \frac{Control\ absorbance - Sample\ absorbance \times 100}{Control\ absorbance}$$

b. Skin-whitening activity-

The method of Tomita et al (1990) was slightly modified.

Preparation of Standard solution- 100mg of ascorbic acid was dissolved in 100 mL of methanol. Dilutions of this solution with 10mL methanol were prepared to give the concentration of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg/mL.

Preparation of test sample- 30mL of methanolic extract was dissolve in 30 mL methanol. Dilutions of this solution with 10mL methanol were prepared to give the concentration of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg/mL.

Preparation of reagents

- Phosphate buffer: 0.2M phosphate buffer of pH 6.8 was prepared according to I.P.
- 23.5mg of L-Tyrosine was dissolve in 25mL of double distilled water.
- 33.1mg of L-Dopa was dissolve in 25mL of double distilled water.
- 0.1ml of H2O2 was dissolved in 10mL of double distilled water.

Protocol of skin-whitening activity

- Different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg/mL) of standard and test solution was used in this method.
- To each of the volumetric flask 1.8mL of 0.2M phosphate buffer (pH 6.8) and 0.6mL of H2O2, 0.1mL of test and standard solution in each concentration and 0.1mL of aqueous solution of L-Tyrosine.
- The mixture was incubated for 5 minutes at 200C in oven.
- In above solution added 0.4mL of 6.3mM L-Dopa.
- Absorbance was recorded at 476nm.

The percentage inhibition of anti-tyrosinase method was calculated by using equation no.3-

$$\% \text{ inhibition} = \frac{(A - B)}{A} \times 100$$

Where

A = absorbance at 476 nm without the test sample, B=absorbance at 476nm with the test sample.

FORMULATION DEVELOPMENT OF METHANOLIC EXTRACT OF CLOVE-

1. Preparation of oil Phase- Cetyl alcohol and steric acid were added together in chine dish and liquefied on hot plate. The methanolic extract was added in this phase after liquefaction of oily phase.

2. Preparation of aqueous Phase-Distilled water, Potassium hydroxide, Methyl paraben, and propyle paraben were mixed together.
3. Oil phase was added in aqueous phase with continues starring until homogenous of formulation and packed.

Table1 – Composition of cream

S.no	Ingredients	Formula w/w %				
1	Cetyl alcohol	F1	F2	F3	F4	F5
		1	2	3	4	5
2	Steric acid	15	14	13	12	11
3	Methyl paraben	0.2	0.2	0.2	0.2	0.2
4	Propyl paraben	0.2	0.2	0.2	0.2	0.2
5	Glycerin	10	10	10	10	10
6	Potassium hydroxide	1.2	1.2	1.2	1.2	1.2
7	Methanolic Extract	5	5	5	5	5
8	Distilled water	q.s	q.s	q.s	q.s	q.s

EVALUATION PARAMETERS OF FORMULATIONS CONTAINED METHANOLIC EXTRACT OF CLOVE

a. Physical Evaluations Homogeneity

Homogeneity of the preparation was measured by visually inspecting the formulations for uniformity and by checking the presence of aggregates and uniformity of colour. The homogeneity of semisolids formulation is a primary need.

b. Measurement of pH

The pH of the formulations was measured in order to determine that whether its pH value lies in the pH range of skin or not. For this prepared formulations were taken and the pH was measured using pH meter. The adjustment in pH was done Triethanolamine.

c. Spreadebility

The efficacy of topical therapy depends on the patient spreading the formulation in an even layer to deliver a standard dose. The optimum consistency of such a formulation helps ensure that a suitable dose is applied or delivered to the target site. This is particularly important with formulations of potent drugs. A reduced dose would not deliver the desired effect, and an excessive dose may lead to undesirable side effects Garg et al (2002).

The Spreadebility was calculated by using the equation no.4. in gm.cm/sec

$$S= m\times L\ T$$

Where,

S, is the Spreadebility of cream formulations M, is the weight (g) tied on the upper plate, L, is the length cm) of the glass plates, and T is the time taken for plates to slide the entire length.

The parallel plate method is the most widely used method for determining and quantifying the Spreadebility of semisolid preparations. The advantages of the method are simplicity and relative lack of expense.

d. Viscosity

Viscosity is defined as an index of resistance of a liquid to flow. The higher the viscosity of the liquid, the greater is the resistance to flow. Viscosities of the formulations were measuring the yield value using Brookfield viscometer.

e. In vitro permeation test of formulations

Methanolic extract of clove release was determined through rat skin by invert test tube method, and the receptor medium was phosphate buffer with methanol (1:1) in a beaker Nikunjana (2009). The amount of the formulation was taken 1gm in a test tube and medium was 100mL in a beaker. The temperature of the medium was maintained 37±1 0C and samples were collected in a 30min of interval for 5 h. The absorbance of samples was taken by UV spectroscopy and all experiments were repeated 3 times. The percentage of cumulative drug release was determined.

f. Permeation test

The whole project was approved with institutional animal ethical committee. In the skin irritation method rats were used as animal modules for skin irritation test. Rats of weight between200-300gm were used for testing of skin irritation. The animals were divided into two groups, one group was control and another was test. The animals were maintained on standard animal feed and allow free access to water. The animals were kept under standard conditions. Hairs were removed from back of rats and area 2cm2 was marker on both sides. One side served as control while the other side was test. On the test side final formulation containing combination of extracts from clove whereas without extract use control. Emulsion was applied (500mg/rats) twice a day for 7days and the site was observed for any sensitivity and the reaction if, any was graded as 0, 1, 2, 3 for no reaction.

g. Stability

It was recommended that samples for the evaluation of stability be placed in neutral, transparent glass flasks with a lid that assured good closing, avoiding gas or vapor losses to the environment. The quantity of product was must be sufficient for the necessary appraisals. In the case of a known incompatibility between the components of the formulations and glass, must choose another containing material. The incorporation of air in the product must be avoided during placement in the test recipient. It was important not to fill the total volume of the package, allowed a head space of approximately one third of the capacity of the flask for possible gaseous exchanges. The final containing material was used parallel to the neutral glass thus anticipating the appraisal of compatibility between the formulation and the packaging material. The duration of the study was generally fifteen days and helps in the screening of the formulations. Stability of the formulations was assessed by using humidity chamber on condition temperature 400C /75RH.

RESULTS

EVALUATION PARAMETERS OF METHANOLIC EXTRACT OF CLOVE

a. Antioxidant activity-

1. DPPH free radical method-

The DPPH free radical method was used for antioxidant activity of methanolic extract of clove. The percentage inhibition of standard solution on 500µg/mL give 84.21% while test solution given 61% on

same concentration. The Table no.3.2 show the percentage inhibition between standard and test solution and the figure no.3.1 shown the graph of percentage inhibition between standard and test solution.

Table 1.1- Percentage inhibition of DPPH method between standard and test solutions

S.NO.	CON (µg/mL)	% INHIBITION OF STANDARD	% INHIBITION OF TEST
1	50	29.26	10.7
2	100	34.62	16.79
3	150	41.84	22.38
4	200	48.02	29.6
5	250	54.62	35.09
6	300	61.45	42.18
7	350	69.91	48.1
8	400	76.41	53.07
9	500	84.21	61

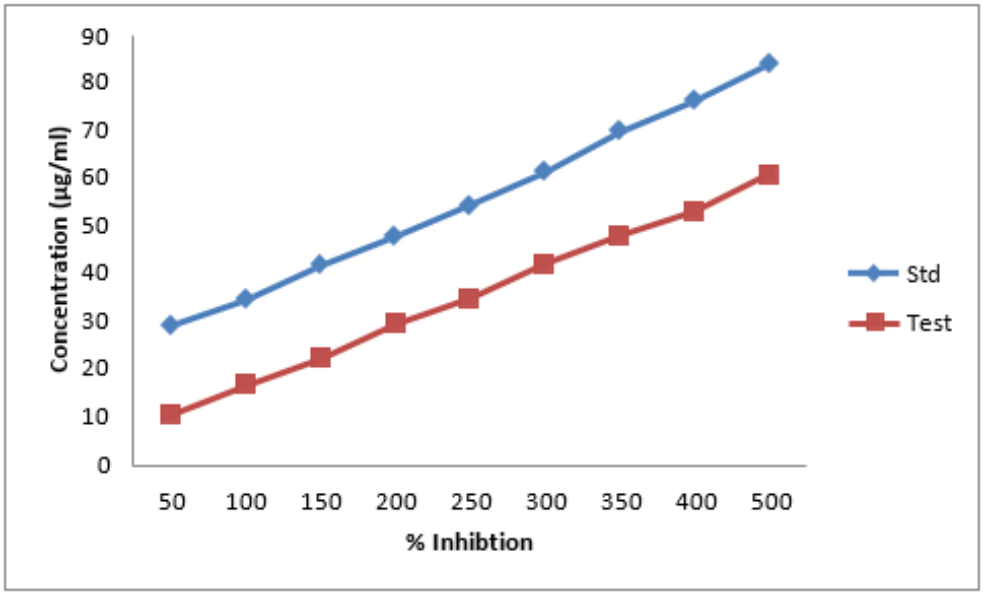


Figure 2.1- Graph of Percentage inhibition of DPPH method between standard and test solution

2. Ferric reducing antioxidant power (FRAP) method

The ferric reducing antioxidant power was also used for antioxidant activity of methanolic extract of clove. The result was come out of percentage Inhibition between standard and test solution shown on table no.3.3 and comparative graph of percentage inhibition between standard and test shown in figure no.3.2. The standard was given percentage inhibition on 500µg/mL 78.28% and test solution given 25.47% on same concentration.

Table 2.2 - Percentage inhibition of ferric reducing antioxident power method between standard and test solution

S.NO.	CONCENTRATION (µg/mL)	%INHIBITION OF STANDARD	% INHIBITION OF TEST
1	50	70.77	2.14
2	100	71.04	4.76
3	200	72.65	9.52
4	300	73.99	13.15
5	400	78.28	19.7
6	500	78.28	25.47

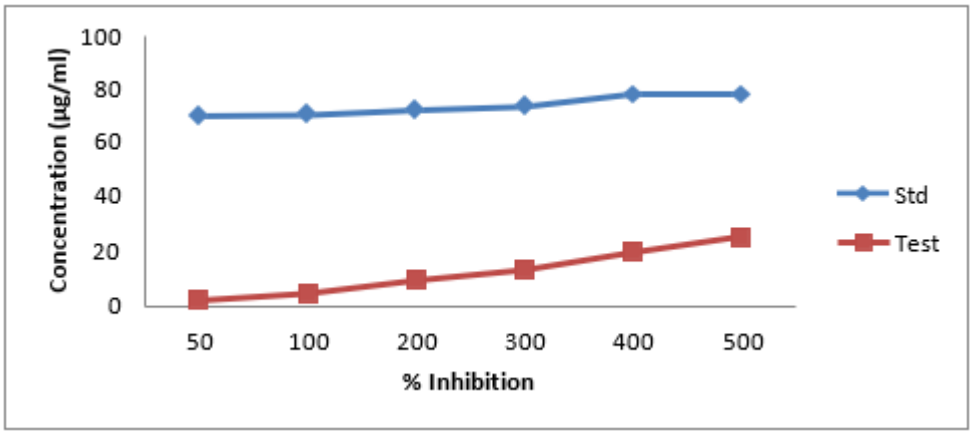


Figure 2.2 - Percentage inhibition graph between standard and test by ferric reducing antioxidant power method

b. Skin-whitening activity of methanolic extract of clove

The methanolic extract of clove was show antityrosinase activity. The result of comparatively study of percentage inhibition between standard and test solution was shown on table no.3.4 and comparatively graph of percentage inhibition between in figure no.3.3.The result of antityrosinase activity of standard on 100µg/mL given 86.28% and test sample given 52.65% on same concentration.

Table 2.3- Antityrosinase activity of standard and test solution

S.NO	CONCENTRATION (µg/mL)	% INHIBITION OF STANDARD	% INHIBITION OF TEST
1	10	40.24	1.38
2	20	45.86	5.79
3	30	51.27	11.23
4	40	57.62	16.55
5	50	61.23	22.13
6	60	65.24	27.85
7	70	70.12	35.65
8	80	75.27	42.19
9	90	81.11	47.34
10	100	86.28	52.65

EVALUATION OF FORMULATION

A. Physical evaluation

Determination of Physical appearance

The color was observed visually. The average of three reading was recorded. The physical appearance of the formulations is shown in table no.3.6-

Table 3.1- Physical appearance of formulations

F1	**F2**	**F3**	**F4**	**F5**
Off white	Off white	Off white	Yellowish	White

B. pH of formulations-

pH of the formulations was determine as there is an essential need to prepared a formulations which is compatible to be used on a skin without any adverse effect. So to check the compatibility of the formulations with the skin it was assessed that whether the pH of the formulations come in range of skin (4.5-7) or not by using digital pH meter. The result is shown in table no.3.7 from which it can be concluded that all formulations have pH to be used on skin. The pH of the formulations, were found immersing pH meter to a depth 0.5 cm in a beaker containing formulations. The determinations were carried out in triplicate and the average of three reading was recorded.

Table 3.2- pH of the formulations

F1	**F2**	**F3**	**F4**	**F5**
6.4±0.3	6.8±0.3	6±0.2	6.6±0.21	5.5±0.1

c. Determination of Spreadability :

The Spreadability of the formulations was determining by parallel plate method. The result of Spreadability is shown in table no.3.8-

Table 3.3-Spreadebility (gm.cm/sec) of formulations

F1	**F2**	**F3**	**F4**	**F5**
1.77±0.88	0.74±0.11	0.48±0	0.88±0.08	0.38±0.05

d. Viscosity

The viscosity of formulated semisolids bases was determined. The viscosity determinations were carried out on Brook-field viscometer using spindle number S-06 and the average of nine reading is recorded and given in table no.3.9-

Table 3.4- Viscosity (cps) of formulations

F1	F2	F3	F4	F5
1155±1.2	1729±0.57	4386±0.58	5088±0.55	4766±0.33

e. In vitro Permeation study-

Methanolic extract of clove release was determined through rat skin by invert test tube method, and the receptor medium was phosphate buffer with methanol (1:1) in a beaker. The amount of the formulation was taken 5gm in a test tube and medium was 100 mL in a beaker. The temperature of the medium was maintained 37±1 0C and sample collected in a 30min of interval. The absorbance was taken by UV spectroscopy. The percentage of cumulative drug release was found out and given in followings tables no

Figure 1.- Invert test tube method for In-Vitro release of formulations

Table 3.5- In-vitro drug release of formulations

TIME (min)	**% Cumulative drug release**				
	F1	F2	F3	F4	F5
0	0	0	0	0	0
30	3.11	2.46	0.16	5.9	9.35
60	4.23	3.36	1.216	11.73	11
90	10.2	4.82	3.25	14.33	15.22
120	17.4	11.97	6.67	16.9	18.19
150	26.54	21.92	15.4	18.62	21.98
180	31.01	26.19	26.28	29.06	27.98

210	34.39	37.26	34	33.85	34.13
240	50.14	42.33	42.8	45.93	44.72
270	58.52	59.55	51.6	62.57	52
300	68.32	68	60.7	71	81.6

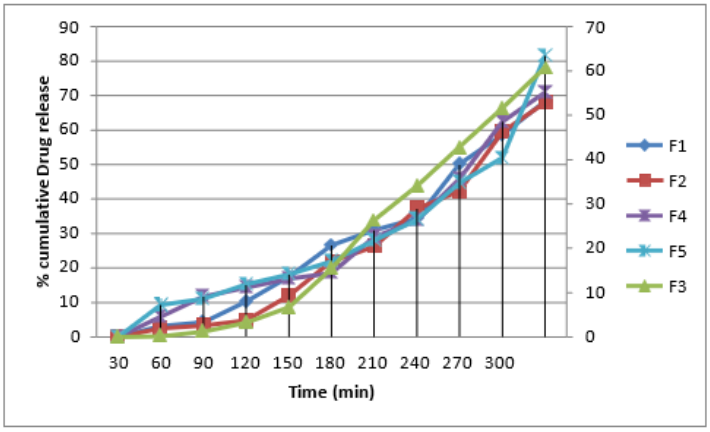


Fig. 2.4 Graph of cream formulation drug release

Comparison of the in-vitro drug release of the formulations demonstrated that the rank order was approximately the same, F5 > F4 > F2 > F1 > F3, for all combinations. The results of the formulations F1and F2 were found similar. The lowest release was showed F3 and the maximum release was showed in F5 formulation. The highest release profile was showed by F5.

f. Stability test of formulations

The stability studies of formulations were carried out under the following condition 40/75(0C/RH) for one month. The effects of temperature, humidity and time on the physical characteristics of the creams were for assessing the stability of the prepared formulations.

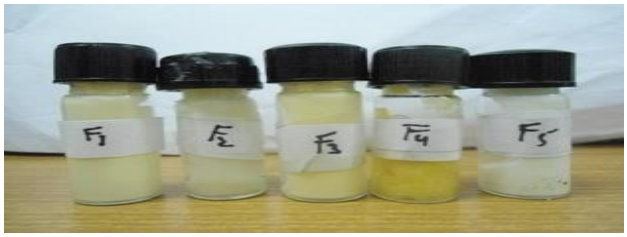
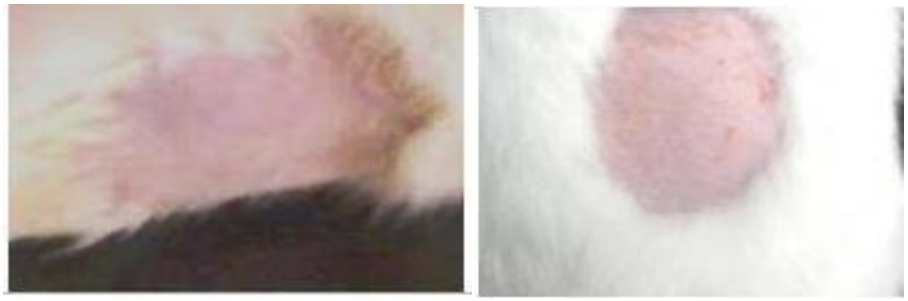


Figure 3.10- Formulations for skin whetining

EVALUATION PARAMETER FOR FINAL FORMULATION

a. Skin irritation testing on Rat

The skin irritation test of the final formulation was performed on rat. The animals were divided into two groups control and test. After the application of final formulation on rat skin was not found any type of erythema or any other skin sensitivity reaction. The result was found that the application of formulation completely safe on skin



Control Test

Figure 3.12- Skin-irritation test on rat skin

PARAMETER	INITIAL	AFTER ONE MONTH 40^{0} C 75 RH
Appearance	White	White
Feel on application	smooth	Smooth
pH	5.5 ±0.1	5.7 ±0.3
Viscosity	47676 Cm Poise	47679 Cm Poise
Spreadebility	0.38±0.5	0.38±0.5
Skin-irritation	Non-irritant	Non-irritant
Homogeneity	Homogeneous	Homogeneous
Stability	Stable	Stable

The selection of best formulation was based on in-vitro release profile, non-irritant property and stability on 450C.

SUMMARY AND CONCLUSION

The present study was designed in order to formulate a herbal formulation of methanolic extract of clove for skin whitening activity. The plant Syzygium aromaticum was used in the formulation of cream.

The methanolic extract of clove was used as active ingredient for development of formulations. Different types of formulation (Vanishing cream, o/w emulsion and multiple emulsions w/o/w) were prepared by using 5% of methanolic extract of Syzygium aromaticum as skin whiting ingredient. The various parameters (pH, viscosity, homogeneity, Spreadebility, skin-irritation, in-vitro release and stability) of formulation were evaluated.

Comparison of the in-vitro of the formulations demonstrated that the rank order was approximately the same, F5 > F4 > F2 > F1 > F3, for all combinations. The results of the formulations F1and F2 were found similar. The lowest release was showed F3 and the maximum release was showed in F5 formulation. The highest release profile was showed by F5. The parameters of formulation were evaluated and on basis of that best formulation was selected. The formulation was found non-irritant to the skin and showed satisfactory result for the physico-chemical parameters such as pH, spreadability, homogeneity and viscosity. The formulation was also found to be stable on 450C temperature.

Thus from the above study we can establish that the formulation containing herbal ingredients can be used effectively to protect against the harmful effect of skin pigmentation as wall as inhibition of tyrosinase which provided skin-whitening effect. Further studies can be done in order to isolate the active constituent of the plant which is responsible for showing skin- whitening activity.

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