Development of a Herbal Kajal Formulation: A QbD-Based Approach with Gas Chromatographic Profiling of Vitamins A & E and HET-CAM Safety Evaluation

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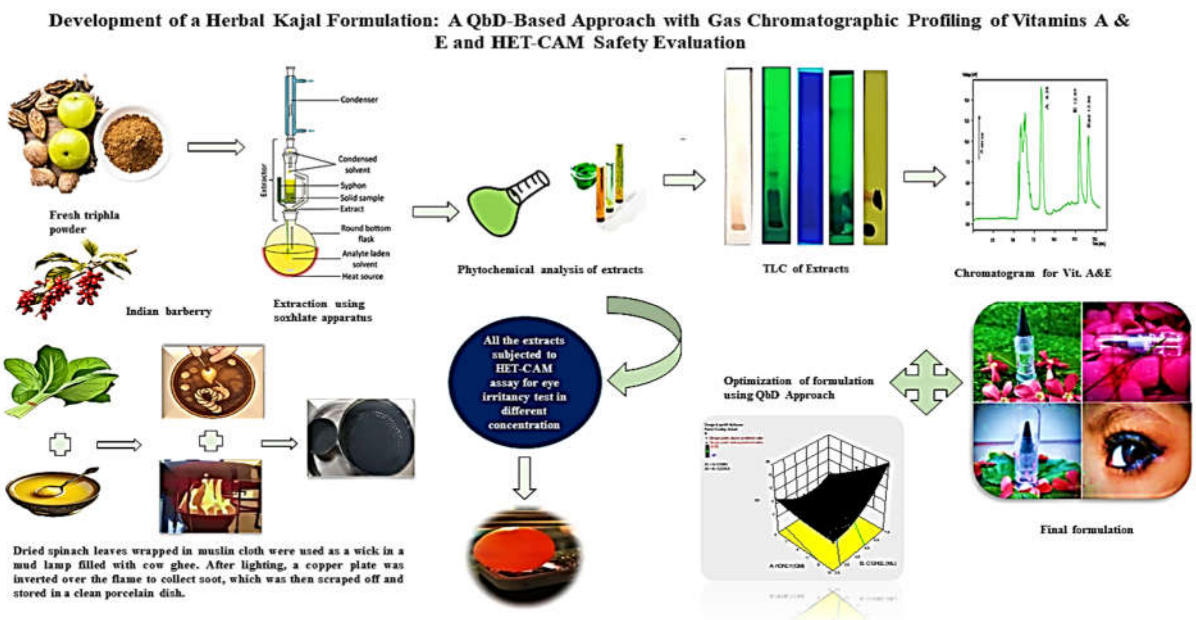
Graphical Abstract



Abstract:

Background: The present study focuses on formulation and evaluation of a novel herbal kajal using a Quality by Design (QbD) approach, integrating traditional herbal knowledge with modern analytical and safety techniques.

Purpose: The purpose was to optimize formulation parameters, confirm the presence of fat-soluble vitamins, and assess ocular safety.

Study design: Experimental study with formulation development, GC analysis, and HET-CAM safety evaluation.

Methods: Critical material attributes and process parameters were optimized using QbD methodology. Gas Chromatography confirmed vitamin A and E presence. Safety was evaluated using the Hen's Egg Test on the Chorioallantoic Membrane.

Results: GC analysis confirmed the presence of retinol and α-tocopherol. HET-CAM assay demonstrated the formulation is non-irritant.

Conclusion: The developed herbal kajal is a natural, safe, and effective alternative to synthetic cosmetics, suitable for further clinical evaluation and scale-up.

The present study focuses on formulation and evaluation of a novel herbal kajal developed using a Quality by Design (QbD) approach to ensure consistent quality, efficacy, and safety. The QbD methodology allowed for the identification and optimization of critical material attributes (CMAs) and critical process parameters (CPPs) that influence the final product. Carefully selected herbal ingredients known for their ocular benefits underwent scientific validation of their phytochemical composition. To evaluate the nutritional and therapeutic content of the formulation, Gas Chromatography (GC) analysis was performed, confirming the presence of essential fat-soluble vitamins, specifically Vitamin A and Vitamin E, which are known for their antioxidant and eye-protective properties. A safety assessment was conducted using the Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM), demonstrating that the formulation is non-irritant and safe for ophthalmic use.The integration of traditional herbal knowledge with modern pharmaceutical development techniques highlights the potential of this herbal kajal as a natural, safe, and effective alternative to synthetic eye cosmetics. This study lays a foundation for further development, scale-up, and clinical evaluation of herbal eye formulations guided by scientific principles.

Keywords: Herbal Kajal, QbD, Gas Chromatography, Eye Irritancy test, HET-CAM,Vitamin A&E,

Abbreviations: CMA, Critical Material Attributes, CPPs: Critical Process Parameters, DOE: Design of Experiments, QbD: Quality by Design, GC: Gas Chromatography, HET-CAM: Hen's Egg Test on the Chorioallantoic Membrane,

1. Introduction

Traditionally known as Kajal, Surma, or Kohl, this eyeliner holds a significant place in Indian culture, valued for both its cosmetic appeal and therapeutic benefits for eye health. The innovation of developing Ayurvedic Kajal using medicinal plants reflects a progressive blend of traditional wisdom and modern cosmetic science. In Vedic philosophy, the eyes are considered a vital organ, serving as a bridge between the inner and outer worlds, and natural eye care methods are widely promoted to enhance both beauty and health. **(Waghulde et al., 2018), (Varpe et al., 2022).** Kajal, typically applied to the inner rims of the eyelids, is a thick black ointment traditionally made with ingredients like ghee and spinach, which support eye health. Our formulation is inspired by these practices, incorporating Triphala a blend of *amla*, *bibhitaki*, and *haritaki*—well-known in Ayurveda for promoting vision and ocular well-being. Cow ghee serves as a soothing base, rich in omega-3 fatty acids, while spinach contributes essential vitamins A and E. Additional ingredients include Indian barberry to promote blood circulation around the eyes, honey for its anti-inflammatory and antimicrobial effects, and camphor oil for its cooling properties **(Sangale and Bhangale, 2023).** To ensure quality, safety, and efficacy, the Quality by Design (QbD) approach was employed, allowing for systematic optimization of the formulation and manufacturing process. The growing interest in herbal and natural remedies reinforces the importance of QbD in modern herbal product development. **(Khandai et al., 2024).** For safety evaluation, the Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) was conducted. This method, recognized for being sensitive, rapid, and cost-effective, mimics the vascular response of human conjunctival tissue and serves as an ethical alternative to animal testing for assessing ocular irritancy (**Wagh and Shahi 2018), (Kishore et al. 2008).** Analytical validation was carried out using Gas Chromatography (GC), which offers high sensitivity and selectivity for identifying bioactive compounds. GC analysis confirmed the presence of retinol (Vitamin A) and α-tocopherol (Vitamin E) two key fat-soluble antioxidants known for their roles in eye protection, anti-inflammatory activity, and skin health. Though GC is more suited to thermally stable, volatile compounds, its application here effectively validated the nutritional and therapeutic value of the formulation **(Qian et al. 2017).** In summary, this work highlights a holistic approach to eye care through a carefully developed Ayurvedic Kajal, rooted in traditional knowledge and supported by modern analytical and safety techniques. The synergistic effects of its natural ingredients, especially Triphala and Vitamin E, reflect its dual function as a cosmetic and therapeutic product.

2. Material and Method

2.1 Collection of Plant Material

The plant materials—fresh amla (*Phyllanthus emblica*), baheda (*Terminalia bellirica*), haritaki (*Terminalia chebula*), fresh spinach (*Spinacia oleracea*), Indian barberry (*Berberis aristata*), ghee, camphor, and honey—were procured from a reliable local market in Ahmedabad, Gujarat, India, in August 2024. Authentication was carried out by a qualified botanist at Gujarat College, Ahmedabad, affiliated with Gujarat University. A voucher specimen has been prepared and deposited at the herbarium of Gujarat College for future reference.

2.2 Preparation of Alcoholic Extract of Triphala (AlE) and Indian Barberry

In an efficient extraction process, twenty grams of Triphala powder were extracted using the Soxhlet method with 150 mL of methanol over six runs to ensure a potent extract. The extract was then evaporated under vacuum and dried at 38.4°C **(Puthalath et al. 2015), (Sivasankar et al. 2015).** Meanwhile, twenty-five grams of Indian barberry powder were extracted with 150 mL of n-hexane at approximately 70°C. After filtering the extract and residue, the residue underwent a secondary extraction with methanol. The methanolic extract was evaporated in a water bath for 45 minutes, yielding a high-quality concentrate **(Aliakbarlu et al. 2018). (Fig. 1 &2)**

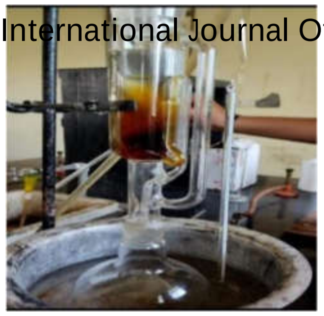


Fig. – 1 Extraction of Triphla & Indian barberry **Fig. – 2 Extraction of Indian barberry**

2.3 Preparation of Ash for the Kajal

One kilogram of fresh spinach was taken and dried. A piece of muslin cloth was used to hold the dried spinach leaves, which then served as a wick in a mud lamp filled with cow ghee. After lighting the lamp, an inverted copper plate was placed on top. The black soot that collected on the plate was scraped off and gathered into a clean, dry porcelain dish **(Arru et al. 2021). (Fig. 3-8)**

Fig. – 3 Fresh Spinach leaves **Fig. – 4 dried Spinach leaves** **Fig. – 5 mud lamp filled with cow ghee**

Fig. – 6 lighting the lamp **Fig.7 plate on lighting the lamp** **Fig. – 8 dry dish contain Ash**

3. Evaluation of Herbal Kajal

3.1 Preliminary Phytochemical screening

The prepared test extracts were subjected to phytochemical screening to detect the presence of alkaloids, glycosides, saponins, fixed oils, phytosterols, phenols, flavonoids, gums, and mucilages, following standard protocols **(Kumar et al. 2017), (Chauhan et al. 2012).** Thin Layer Chromatography (TLC) was used to identify key bioactive markers, including chebulagic acid, gallic acid, and chebulinic acid. The analysis was performed on pre-coated silica gel 60 F254 plates (0.2 mm thickness), using a mobile phase of toluene:ethyl acetate:formic acid (2:5:1.5). Post-development, the plates were derivatized with a 5% ferric chloride solution according to Ayurvedic Pharmacopoeia guidelines, and Rf values were recorded.For Indian barberry extracts, TLC was performed using a mobile phase of n-butanol:ethyl acetate:acetic acid:water (3:5:1:1). The developed plates were analyzed under UV light at 366 nm. Additional phytochemical tests confirmed the presence of the targeted compounds **(Sivasankar et al. 2015).**

3.2 Eye Irritancy Testing

The Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) was employed as a reliable alternative to in vivo methods for evaluating eye irritation potential. Fresh, fertile white Leghorn chicken eggs (50–60 g, ≤7 days old) were procured from a commercial supplier. After candling to exclude nonviable specimens, eggs were incubated in a BOD incubator at 38.4 °C and rotated manually five times daily for eight days. Embryo development was monitored regularly, and by day eight, neural development confirmed readiness for testing. Prior to treatment, eggs were immersed in 0.9% NaCl for optimal conditioning, followed by an additional 30-minute incubation. Test extracts of Triphala and Indian barberry were diluted in NaCl to prepare various concentrations. Post-application, embryos were incubated for 30 minutes and observed for signs of cell lysis, haemorrhage, and coagulation. All observations were documented thoroughly for analysis. This protocol highlights the effectiveness of the HET- CAM assay in assessing the ocular irritation potential of herbal extracts in a controlled, ethically sound manner **(Wagh and Shahi 2018), (Kishore et al. 2008) (Fig. 9&10).**

Fig. – 9 eggs were placed in a BOD incubator

Fig. – 10 egg after incubation

3.3 Viscosity, Spreadability, optimization by using QbD approach

Design of Experiments (DOE) was employed to explore the quadratic response surface and develop a robust polynomial model using Design-Expert software (Trial Version 7.0.2, Stat-Ease Inc., MN). A comprehensive factorial design was implemented to optimize two key independent variables: the honey-to-mix ratio (X1) and ghee content (X2), with in vitro drug release as the primary dependent variable. This design allowed for the evaluation of main, interaction, and quadratic effects. Formulation preparation involved dissolving Triphala and Indian barberry extracts in accurately weighed honey, while ash was separately mixed into ghee. These two mixtures were then combined, followed by the addition of camphor oil to ensure uniform blending. The final formulation was evaluated for viscosity and spreadability using an LMDV-60 rotational viscometer at 60 RPM and a shear rate of 74.5%. This systematic approach ensured optimized formulation characteristics with reliable quality and performance outcomes **(Shah et al. 2016), (Shah and Patel 2025), (Jain et al. 2021).**

3.4 Identification of Vit. A and E through Gas Chromatography (GC)

The analytical testing was conducted to identify and assess the effectiveness of vitamins A and E, along with residual solvent testing. We utilized the Agilent 7890A GC equipped, a crucial instrument for our analysis. A 0.5000 g sample of a well- homogenized cereal product was diluted in 1 mL of ethanol and left overnight in the dark. After thorough mixing with hexane and dichloromethane (90%/10% v/v), the solution was centrifuged at 2500 rpm for 10 minutes, and the organic layer was evaporated at 40°C. The sample was then re- dissolved in 1 mL of ethanol, and 2 µL was injected into the GC system. This procedure was also applied to the standards **(Krishna et al. 2013)**, **(Vasim et al. 2016)**. Analytical conditions:

Column: Agilent HP-1 (30m, 0.32mm, 0.25mm) Injection Volume: 2mcl, Spitless mode.

Injector temperature:300°C

Carrier gas: Helium at a flow rate of 2.0ml/min

Detector Temp. 340 °C (The initial oven temperature, 120°C, was held for 1 min and was then meticulously programmed at 27°C/min to 320°C, which was held for 15 min.)

GC method for determination of residual solvent **(Table-1)**

Sample Preparation (Standard vit. A&E and Sample Spinach Extracts)

Diluent: DMSO (Dimethyl Sulphoxide)

Standard Preparation: 0.3ml of Methanol diluted to 50ml with DMSO and further 5ml of this solution was diluted to 50ml with diluent.

Sample Preparation: 100mg sample diluted with 1 ml of DMSO in a 20 ml HS vial

pH of the Ash Paste: 0.5gm of sample take in 5 ml of water and mixed well. pH of the solution was measured by pH meter.

Column	DB-624 (30M*0.53 mm*µm)
Flow	2ml. min.
Injection volume	HEADSPACE
Injection Temperature	170°C
Detector Temperature	250°C
Split Ratio	1:10
Run Time	33 min
Vial Oven Temperature	90°C
Loop Temperature	100°C
Transfer line Temperature	110°C
GC Cycle	48 min
Vial Equilibration time	15 min

Table -1 (HS-GC) Headspace gas chromatography parameters

4. Results

4.1 collection of plant material

The plant materials—fresh amla (*Phyllanthus emblica*), baheda (*Terminalia bellirica*), haritaki (*Terminalia chebula*), fresh spinach (*Spinacia oleracea*), Indian barberry (*Berberis aristata*), ghee, camphor, and honey were procured from a reliable local market in Ahmedabad, Gujarat, India, in August 2024. Authentication was carried out by a qualified botanist at Gujarat College, Ahmedabad, affiliated with Gujarat University. A voucher specimen has been prepared and deposited at the herbarium of Gujarat College for future reference.

4.2 Preliminary Phytochemical screening

Thin Layer Chromatography & chemical test was performed for the Identification of the bio-actives such as chebulagic acid, gallic acid & chebulinic acid present in the plant extracts, which we can see in the image.

Rf value calculated such as 0.4, chebulagic acid 0.5 chebulinic acid, and 0.2 gallic acid. **(Fig. 11) (Table-2) (Fig. 12)** The Rf Value of barberine was found to be 0.55. The Chemical test was performed confirm the present of various phytochemicals. Phytochemical analysis of triphla and indian barberry extracts confirmed by the literature **(Krishna et al. 2013), (Vasim et al. (2016). (Fig. 13) (Table-3).**

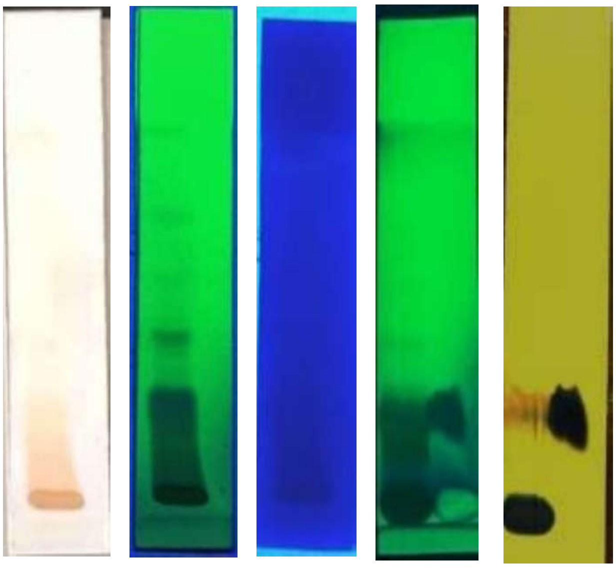


Fig. –11 TLC of Triphla powder with gallic acid

Fig. 12- Chemical test of Gallic Acid

Sr. No.	Test	Observation	Result	Inference
1.	1ml aq. Extract + 2ml of 5% ferric chloride	Dark Blue colour or greenish colour	Positive test	Gallic acid present.
2.	1ml solution of extract + 2.5 of gelatin solution	Yellowish colour	Positive test	Gallic acid present.

Table - 2 Chemical Test of Gallic Acid

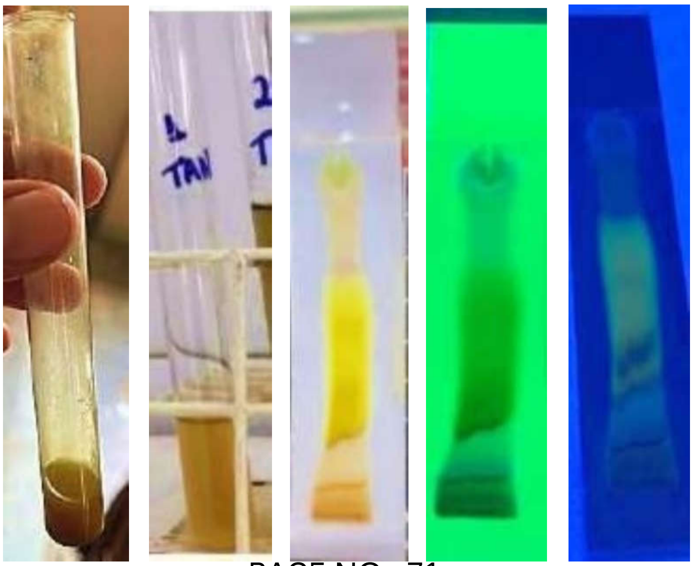


Fig. 13- Chemical test & TLC Indian of barberry

Sr. No.	Test	Observation	Result	Inference
1.	Berberine solution + Con.HNO_3	Reddish brown colour	Positive test	Berberine Present
2.	Berberine solution + Con.H_2SO_4	Orange – Yellow colour	Positive test	Berberine Present
3.	Berberine solution + a drops of Bromine water + few drops of HCL	Bright – Red colour	Positive test	Berberine Present

Table-3 Chemical Test of indian barrabry powder

4.2 Eye Irritancy Testing

Treatment of eggs with extracts of different amount to check the irritancy level in the eye for that HET- CAM test method used. Fresh fertile white Leghorn chicken eggs were obtained from commercial sources, not older than seven days with weighing between 50 & 60 gm were used and eggs were candled prior to use and nonviable or defective eggs were discarded. As per the method, Fertilized eggs were incubated in a BOD Incubator at temperature 38.4°C and eggs were hand rotated 5 times until day 8. All experiments were performed in triplicate. The results are presented as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism software, and differences were considered statistically significant at $p < 0.05$. **(Table-4) (Fig. 14) (Table-5) (Fig. 15).**

Fig. -14 Eye irritancy analysis of Triphala Extract amount 5,7,10,12,15mg

Sample (mg)	Replicate 1	Replicate 2	Replicate 3	Result Summary
5 mg	Negative	Negative	Negative	No irritation observed

7 mg	Negative	Negative	Negative	No irritation observed
10 mg	Negative	Negative	Negative	No irritation observed
12 mg	Negative	Negative	Negative	No irritation observed
15 mg	Negative	Negative	Negative	No irritation observed

Table-4 Eye irritancy analysis of triphala extracts in different concentration

Fig. -15 Eye irritancy analysis of Indian Barberry extracts amount 5,7,10,12,15mg

Sample (mg)	Replicate 1	Replicate 2	Replicate 3	Result Summary
5 mg	Negative	Negative	Negative	No irritation observed
7 mg	Negative	Negative	Negative	No irritation observed
10 mg	Negative	Negative	Negative	No irritation observed
12 mg	Negative	Negative	Negative	No irritation observed
15 mg	Negative	Slight Redness	Negative	Minimal irritation observed in one replicate

Table- 5 Eye irritancy analysis of Indian Barberry extracts in different concentration

4.3 Viscosity, Spreadability and optimization of formulation

All experiments were performed in triplicate. The results are presented as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism software, and differences were considered statistically significant at $p < 0.05$. as per **(table 6).**

Formulation No.	Honey (g)	Ghee (ml)	Viscosity (cP)	Spreadability (gm.cm/s)
1	10	7.5	14900 ± 150	12.5 ± 0.3
2	15	5	20450 ± 200	9.4 ± 0.2
3	10	2.5	20740 ± 250	5.4 ± 0.1
4	5	7.5	8600 ± 100	3.8 ± 0.2
5	15	2.5	20600 ± 180	4.7 ± 0.1
6	5	5	20150 ± 190	2.0 ± 0.2
7	10	5	20580 ± 210	3.1 ± 0.1
8	5	2.5	20400 ± 160	7.5 ± 0.3
9	15	7.5	18100 ± 170	18.8 ± 0.5

Table. No. 6 Viscosity and Spreadibility

Quantities of honey and cow ghee were added in the software to find the appropriate formula of formulation on the basis of viscosity and spreadability. (Table-6), Graphical representation of QbD Approach **(Fig. 16)** **(Table-7).**

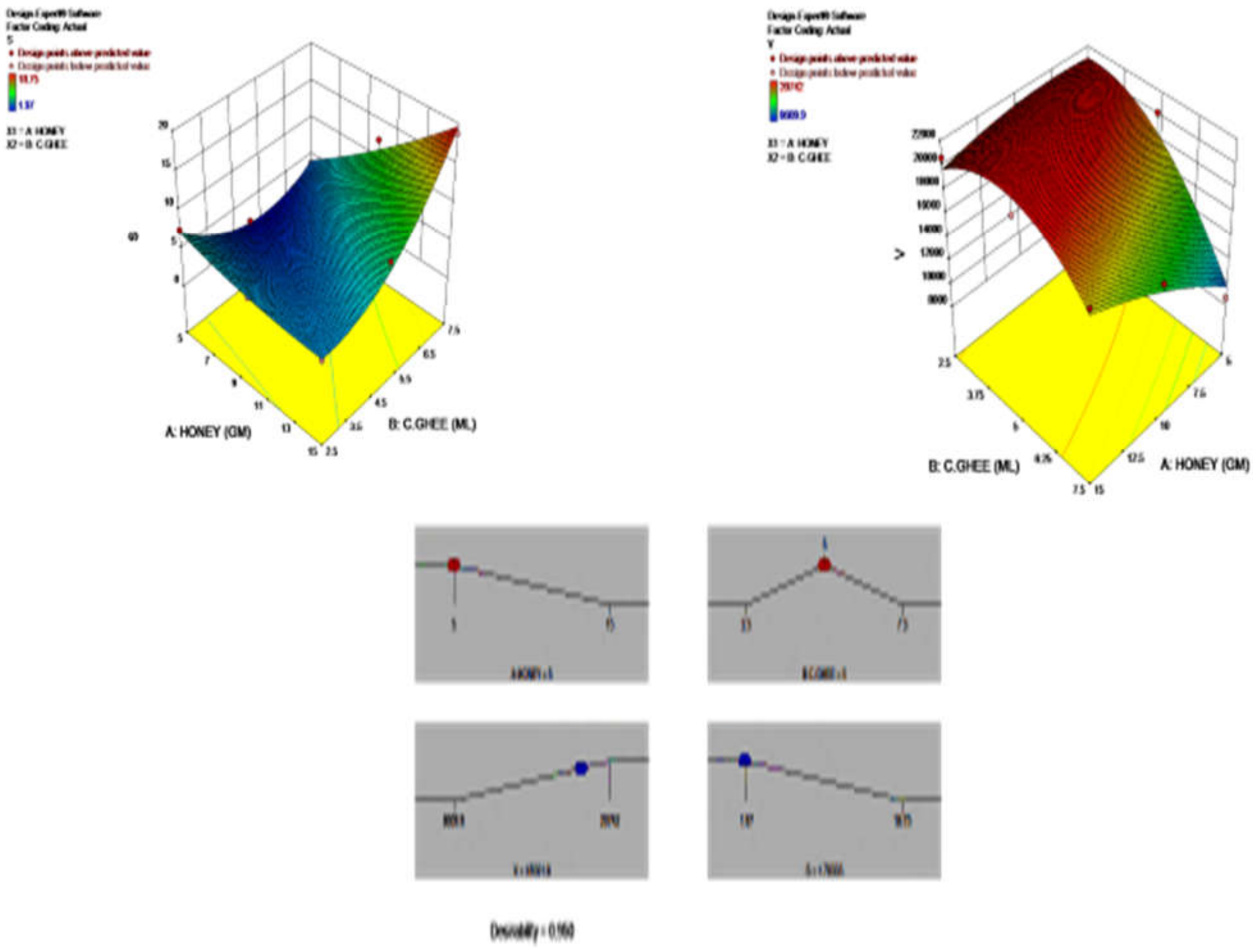


Fig. 16 – QbD analysis result

Sr. No.	Triphala Extract (mg)	Indian barberry extract (mg)	Honey (gm)	Ash (gm)	Ghee (ml)	Camphor Oil (Drops)	Spreadibility gm.cm/s
1	50	50	10	1.5	7.5	10	12.5
2	50	50	15	1.5	5	10	9.37
3	50	50	10	1.5	2.5	10	5.35
4	50	50	5	1.5	7.5	10	3.75
5	50	50	15	1.5	2.5	10	4.68
6	50	50	5	1.5	5	10	1.97
7	50	50	10	1.5	5	10	3.12
8	50	50	5	1.5	2.5	10	9.37
9	50	50	15	1.5	7.5	10	5.35

Table-7 Optimization data of QbD

4.4 Gas Chromatography analysis report

Gas chromatography was employed to detect and confirm the presence of fat-soluble vitamins, specifically vitamin A (retinol) and vitamin E (α- tocopherol), in the prepared formulation. The analysis was conducted without derivatization, and the chromatogram exhibited multiple well-resolved peaks. Among these, two major peaks were observed at retention times of 8.665 minutes and 8.922 minutes, respectively. Based on established literature values and comparison with previously reported chromatographic data, the peak at 8.665 min is tentatively identified as vitamin A (retinol), while the peak at 8.922 min is attributed to vitamin E (α-tocopherol). These peaks demonstrated significant height and sharp resolution, indicating their abundant presence in the sample matrix **(Smidt et al. 1988), (Sheppard et al. 1962) (Fig. 17).**

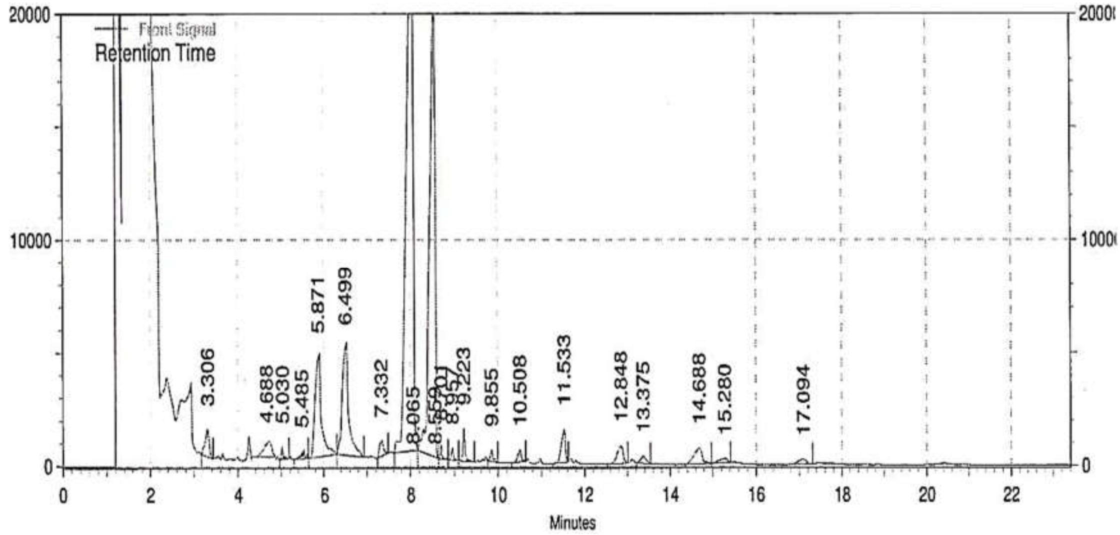


Fig. -17 Gas Chromatogram of vitamin A&E

5. Conclusion:

The present study successfully formulated and evaluated a herbal kajal using the Quality by Design (QbD) approach, ensuring product quality, efficacy, and safety from the development stage. By identifying and optimizing critical material attributes and process parameters, the formulation showed high consistency and reproducibility. The selected herbal ingredients, rich in bioactive compounds, were confirmed via Gas Chromatography (GC) to contain Vitamin A and Vitamin E—nutrients known for their antioxidant, anti-inflammatory, and vision-supportive properties. Safety was assessed using the Hen's Egg Test on the Chorioallantoic Membrane (HET- CAM), which showed negligible irritancy, confirming the product's suitability for periocular use. The GC analysis confirmed the presence of retinol and α-tocopherol, with retention times matching literature values, further supporting the formulation's therapeutic potential. In conclusion, the QbD-based development validated the traditional use of herbal ingredients and established the kajal as a natural, safe, and effective alternative to synthetic eye cosmetics. Future studies may explore stability, scale-up, and clinical evaluation to enhance market readiness.

Footnote: The research work was primarily carried out at Smt. S.M. Shah Pharmacy College during the academic year 2023–2024. The author is currently affiliated with Sal College of Pharmacy, Gujarat Technological University, Gujarat India.

Acknowledgement:

we express our heartfelt gratitude to Sharda School of Pharmacy, Pethapur, for providing financial assistance under the Student Startup and Innovation Policy (SSIP) scheme, which significantly supported this research project. We sincerely thank Smt. S.M. Shah Pharmacy College, Amsaran, for providing the essential laboratory facilities, resources, and guidance during the execution of the research work in the academic years 2023–2024. We further extend our appreciation to all mentors, faculty members, and colleagues who contributed their valuable time and support throughout the research journey.

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Conflict of interest

The authors declare that they have no known disparate financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Credit of Author Statement

Conceptualization: Priyanka Yadav; Methodology: Priyanka Yadav, Bhumika Sharma; Investigation: Bhumika Sharma, Poojan Shah, Vidhi Panchal, Arti Patel; Writing original draft: Priyanka Yadav; Writing, review & editing: Priyanka Yadav; Supervision: Priyanka Yadav. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.