# Optimization of *Saccharomyces cerevisiae*-Mediated Biotransformation Using Box-Behnken Design for Enhanced Flavonoid Profile: Impact on Luteolin, Quercetin, Kaempferol, Isorhamnetin, and Apigenin Content.

**Prof. Dr. V.R. Ravikkumar***
Professor and Head, Dept. of Pharmacognosy, The Erode College of Pharmacy, Affiliated to The Tamil Nadu Dr MGR Medical University, Chennai, ravisrkumar@yahoo.com

**Dr. Tamboli Ennus Tajuddin[1]**
Associate Professor and Head, Department of Pharmacognosy, Annasaheb Dange College of B Pharmacy, Ashta, District: Sangli, Maharashtra 416301, Affiliated to Shivaji University, Kolhapur, tamboli_et@hotmail.com

**Dr. S. Manodhini Elakkiya[2]**
Associate Professor, Department of Pharmaceutics, Karpagam College of Pharmacy, Othakkalmandapam, Coimbatore-32

**Mrs. Fayejabanu Mohammadsohel Zankhwala[3]**
Assistant Professor, Saraswati Institute of Pharmaceutical Sciences (SIPS), Dhanap, Gandhinagar – 382355, fayejazankhwala@gmail.com

**Mr. Rushikesh Vishwas Lavhate[4]**
Lecturer, Bharati Vidyapeeth's Institute of Pharmacy, Belapar, rushikeshlavhate0081@gmail.com

**Dr. Krishnakumar KL[5]**
Professor, Aditya Bangalore Institute of Pharmacy Education & Research, Bangalore-560064, krishnakumarkl@gmail.com

**Dwarakanath[6]**
Department of Environmental Science, Tumkur University, Tumakuru, Karnataka - 572103, India

**Moidul Islam Judder[7]**
Assistant Professor, Royal School of Pharmacy, The Assam Royal Global University, Betkuchi, Opp. Tirupati Balaji Temple, NH 37, Guwahati - 781035, Assam, India, moonzodder@gmail.com

***Correspondence Author:**Prof. Dr. V.R. Ravikkumar,
Professor and Head, Dept. of Pharmacognosy, The Erode College of Pharmacy, Affiliated to The Tamil Nadu Dr MGR Medical University, Chennai, Perundurai Main Road, Veppampalayam, Erode-638112, Tamil Nadu, India. Email: ravisrkumar@yahoo.com

**Abstract**

**Purpose:** This study investigated the biochemical transformation and optimization of fermentation techniques to enhance flavonoid content in a composite extract derived from *Syzygium cumini* seed, *Mangifera indica* seed, *Momordica charantia* fruit, and *Gymnema sylvestre* leaf, along with an analysis of fermentation kinetics.

**Methods:** The herbal mixture was extracted in hot water and fermented using *Saccharomyces cerevisiae*. A Box-Behnken design was employed to optimize the variables for maximizing flavonoid content. The variables included substrate concentration (40%, 60%, and 80%), inoculum volume (2%, 5%, and 8%), and incubation time (40 h, 50 h, and 60 h). Additionally, the process kinetics were evaluated by measuring physical parameters during fermentation.

**Results:** Flavonoid levels, specifically Luteolin, Quercetin, Kaempferol, and Isorhamnetin, were positively influenced by all three variables. However, Apigenin content exhibited a negative correlation with incubation time, with higher durations resulting in reduced Apigenin levels.

**Conclusion:** Flavonoid content was significantly impacted by the interaction between variables, as demonstrated by contour plots. The actual flavonoid yields under optimized conditions closely matched the model's predictions, though Apigenin content displayed a more intricate behavior. Additional optimization was accomplished using the derived model equations.

**Keywords:** Flavonoids, *Saccharomyces cerevisiae*, Box-Behnken design, Biochemical transformation, Fermentation kinetics, Herbal extract optimization.

## Introduction

Flavonoids are an important class of natural products, widely found in the plant kingdom. They belong to a class of plant secondary metabolites with a polyphenolic structure. With almost 6000 compounds, flavonoids are indispensable components in various nutraceutical, pharmaceutical, medicinal, and cosmetic applications due to their capacity to modify gene expression and regulate key cellular enzyme functions [1]. Flavonoids have several subgroups, including flavanone, chalcone, flavone, flavonol, aurone, isoflavone, anthocyanin, and flavan-3-ol. Flavones are associated with modifying enzyme kinetics and producing antioxidant benefits, e.g., Luteolin and Apigenin. Flavonols are linked with powerful anti-inflammatory and antioxidant activities, contributing to chronic disease prevention, e.g., Quercetin, Kaempferol, and Isorhamnetin. Anthocyanidins are connected with heart health, antioxidant effects, and help in obesity and diabetes prevention. Flavanones are related to cardiovascular health, antioxidant, and anti-inflammatory activities [2]. Isoflavones are beneficial in lowering the risk of hormonal cancers such as breast, endometrial, and prostate cancers as well as menopausal symptoms. Flavanols are associated with lowering cholesterol and promoting cardiovascular and neurological health. Overall, flavonoid consumption provides preventive effects for cardiovascular diseases, diabetes, cancer, and neurodegenerative diseases, along with promoting weight management, healthier lifestyles, and longevity [3]. In nature, flavonoids provide a defense mechanism for plants. They are responsible for the color of flowers and fruits to attract pollinators and aid in seed and spore germination. Flavonoids also

protect plants from biotic and abiotic stresses such as UV radiation. They act as signal molecules, phytoalexins, detoxifying agents, and offer protection from frost and drought [4]. Biotransformation is the process of chemical modification using a biological system. Though it is sometimes confused with metabolism, biotransformation is a broader term, encompassing chemical reactions beyond metabolism. Microbial biotransformation can perform reactions that are difficult to carry out due to harsh conditions, substrate specificity, stereoselectivity, or regiospecificity. These reactions typically include oxidation, reduction, hydrolysis, condensation, isomerization, formation of C–C bonds, chiral reactions, and reversal of hydrolytic reactions [5]. Syzygium cumini seed**,** Mangifera indica seed**,** Momordica charantia fruit, and Gymnema sylvestre leaf are known for their antidiabetic activities. A preliminary phytochemical screening of their combined extract showed the presence of antidiabetic flavonoids, such as Apigenin, Luteolin, Quercetin, Kaempferol, and Isorhamnetin, along with their corresponding glycosides. Studies have shown that certain strains can convert glycosides into aglycones. Therefore, a biotransformation study was conducted using an extract containing these four plants to enhance the flavonoid profile [6]. Box-Behnken design (BBD), a method for response surface methodology, is used to study the correlation between independent variables and their effects. A full three-level factorial design can be costly in terms of the number of experiments when more than two factors are involved. The Box-Behnken design, which uses three factors and three levels, requires fewer experiments and avoids extreme conditions that might lead to unsatisfactory results. It has been successfully applied in various optimization studies. For example, Ashrafi et al. (2015) optimized the removal of flumequine from aqueous solutions using BBD, and Furqan et al. (2014) applied it to optimize Aceclofenac solid dispersion [7]. A composite aqueous extract containing major constituents of **Syzygium cumini** seed, **Mangifera indica** seed, **Momordica charantia** fruit, and **Gymnema sylvestre** leaf was prepared. The fermentation parameters, including substrate concentration, inoculum volume, and incubation time, were optimized using response surface methodology to maximize flavonoid content [8]. The physical parameters were also evaluated to understand the fermentation kinetics of the strain under these conditions. This study focuses on process design, optimization, and control attributes for the production of flavonoid aglycones from a polyherbal composition. The interdisciplinary approach used in this work harnesses the biochemical potential of the microbial strain to achieve desirable biotransformation, with potential industrial applications [9].

## Materials and Methods

Plant materials (*Syzygium cumini* seed, *Momordica charantia* fruits, *Mangifera indica* seed kernel, *Gymnema sylvestre* leaves) were collected from Ahmedabad, Gujarat, India and authenticated by a taxonomist, Dr. Kher from Ahmedabad, Botanical. All other chemicals were obtained from SD Fine Chemicals Pvt. Ltd., New Delhi, India. Apigenin, Luteolin, Quercetin, Kaempferol, and Isorhamnetin were purchased from S D Fine-Chem Limited, Mumbai, India.

## Preparation of Extracts

All raw materials were physically cleaned, washed with water, and then dried in an oven at 40°C for 3 hours. The materials were then coarsely ground and sieved over a 30# sieve. The retained material was used for extraction purposes. All four ingredients were mixed in equal ratios (500 g each) in a polybag and then packed in a Soxhlet apparatus. The extraction was

carried out for 72 hours using water as a solvent. The extract obtained was concentrated using a rotary evaporator and then dried to yield brown powder [10, 11].

**Preparation of Saccharomyces cerevisiae for Fermentation**

To prepare Saccharomyces cerevisiae for fermentation in the laboratory, the lyophilized or slant culture is first revived by rehydrating in sterile distilled water or nutrient broth or by streaking onto a growth medium like Malt Extract Agar (MEA) or Yeast Peptone Dextrose (YPD) agar. A suitable growth medium such as YPD broth (containing glucose, yeast extract, and peptone) is prepared, sterilized by autoclaving, and used to grow the yeast. The revived culture is then inoculated into the liquid medium and incubated at 28–30°C with shaking at 120–150 rpm for 12–24 hours to achieve exponential growth (OD600 of 0.8–1.0). The seed culture is further scaled up by transferring it into a larger volume of sterile broth and incubating under similar conditions to produce a sufficient quantity of active yeast cells. The resulting culture, either in suspension or harvested biomass, is used as inoculum for the fermentation process, ensuring all steps are conducted under sterile conditions to avoid contamination [12].

**Fermentation of Polyherbal Extract**

*Saccharomyces cerevisiae* was used for fermentation. The lyophilized culture was revived on malt extract agar medium. The media was prepared, autoclaved, and then kept for 24 hours to check its sterility. After 24 hours, it was streaked with the lyophilized culture. The culture was incubated at 28 ± 0.5°C for 24 hours. After 24 hours, the slant culture was transferred to a seed culture. A malt-ex-yeast-ex-glucose-peptone medium was used to prepare the seed culture media. The seed culture media was prepared, kept for 24 hours, and inoculated with the slant media containing the microbial biomass. It was then incubated at 28 ± 0.5°C for 24 hours [13].

**Method Optimization**

The fermentation process was optimized using Box-Behnken design (Design-Expert software, trial version 10, Stat-Ease Inc., Minneapolis, MN, USA), and the best combination of process variables was determined for the desired responses. Optimization (Response Surface Method Designs, e.g., Central Composite, Box-Behnken) aims to find the optimum set-point in the process design space, which can be either a unique optimum or a compromise to meet conflicting demands. A set of 3 variables: substrate concentration, inoculum volume, and incubation time, was selected as independent variables. The contents of 5 different flavonoids were measured as responses.Based on literature and initial trials, the substrate concentration was set at three levels: 40%, 60%, and 80%. The inoculum volume was selected at 2%, 5%, and 8%. Previous studies reported that Saccharomyces cerevisiae could produce 12–14% ethanol in 72 hours; thus, 50 hours was considered as the midpoint, with 40 hours as the lower level (“−1”) and 60 hours as the upper level (“+1”) in the design (Table 1). The experimental design consisted of 17 runs in (Table 2), including 5 replicates at the design center to estimate the pure error sum of squares. All experiments were conducted in randomized order to minimize the influence of uncontrolled variables that could introduce bias. Each experiment was performed in triplicate, and the average yield was recorded as the response (R). The impact of variables on the response was analyzed using regression analysis of the collected data [14, 15, 16].

**Table 1: Independent and Dependent Variables for Three-Factor, Three-Level Box-Behnken Design**

| Independent Variables | | | | |
|---|---|---|---|---|
| **Code** | **Factor Coded and Actual Levels** | **Lower Level** | **Middle Level** | **Upper Level** |
| A | Substrate concentration (%) | 40 (−1) | 60 (0) | 80 (+1) |
| B | Inoculum volume (%) | 2 (−1) | 5 (0) | 8 (+1) |
| C | Incubation time (hours) | 40 (−1) | 50 (0) | 60 (+1) |
| **Dependent Variables** | | | | |
| **Code** | **Factor** | **Constraints** | | |
| R1 | Apigenin content (mg/ml) | Maximum | | |
| R2 | Luteolin content (mg/ml) | Maximum | | |
| R3 | Quercetin content (mg/ml) | Maximum | | |
| R4 | Kaempferol content (mg/ml) | Maximum | | |
| R5 | Isorhamnetin content (mg/ml) | Maximum | | |

**Table 2: Experimental Strategy of Box-Behnken Design Along with the Observed and Predicted Values of Responses**

| Run | A: Substrate Con. (%) | B: Inoculum Volume (%) | C: Incubation Time (hours) | Apigenin Content | Luteolin Content | Quercetin Content | Kaempferol Content | Isorhamnetin Content |
|---|---|---|---|---|---|---|---|---|
| 1 | 80 | 5 | 60 | 6.91 | 9.68 | 12.21 | 11.92 | 10.02 |
| 2 | 40 | 8 | 50 | 9.71 | 8.81 | 9.12 | 9.38 | 8.72 |
| 3 | 60 | 5 | 50 | 8.12 | 8.00 | 9.88 | 8.91 | 7.92 |
| 4 | 60 | 2 | 40 | 6.40 | 7.78 | 8.23 | 8.09 | 7.81 |
| 5 | 80 | 5 | 40 | 7.78 | 8.12 | 8.68 | 8.22 | 8.09 |
| 6 | 60 | 5 | 50 | 8.11 | 8.01 | 10.19 | 9.12 | 8.08 |
| 7 | 60 | 2 | 60 | 6.95 | 8.09 | 10.82 | 9.48 | 8.18 |
| 8 | 40 | 2 | 50 | 5.92 | 8.22 | 8.18 | 8.08 | 8.23 |
| 9 | 60 | 5 | 50 | 8.09 | 8.32 | 9.79 | 8.81 | 8.21 |
| 10 | 40 | 5 | 60 | 7.11 | 8.29 | 10.48 | 9.58 | 8.42 |
| 11 | 60 | 5 | 50 | 8.13 | 8.02 | 9.98 | 8.99 | 8.02 |
| 12 | 80 | 2 | 50 | 7.21 | 8.52 | 9.69 | 9.18 | 8.52 |
| 13 | 60 | 8 | 40 | 10.09 | 7.91 | 8.38 | 8.31 | 7.81 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14 | 80 | 8 | 50 | 9.12 | 9.68 | 10.42 | 10.32 | 9.58 |
| 15 | 40 | 5 | 40 | 6.82 | 8.21 | 7.92 | 8.31 | 8.19 |
| 16 | 60 | 5 | 50 | 8.14 | 8.08 | 10.12 | 9.02 | 8.12 |
| 17 | 60 | 8 | 60 | 8.90 | 9.19 | 11.92 | 11.81 | 9.32 |

(Note: Each value pair in the response columns represents observed (Actual) / predicted values.)

**Extraction from Fermented Polyherbal Extract**

After completing the fermentation process, the biomass was mixed with a 10 times volume of a 1:1 mixture of aqueous methanol and filtered through 200# nylon cloth. The resulting extract was made methanol-free.

## Results and Discussion

A composite extract containing major constituents of *Syzygium cumini* seed, *Mangifera indica* seed, *Momordica charantia* fruit, and *Gymnema sylvestre* leaf was prepared. The preliminary phytochemical screening showed the good presence of flavonoids in the extract. To enhance the flavonoid profile, it was fermented using *Saccharomyces cerevisiae* strain. The fermentation parameters, i.e., substrate concentration, inoculum volume, and incubation time were optimized to yield maximum content of flavonoids. The experiments were designed, and based on the data obtained from suggested run experiments, model data were derived. For all five responses, the quadratic model was suggested. $R^2$ values were between 0.9999 and 0.9791. The difference between predicted and adjusted $R^2$ values was within the range of 0.0005–0.0949. PRESS characteristics were within 0.0167–0.8725, indicating predictability of individual quadratic models. Adequate precision values were within 19.5839–269.2940.

As shown in Table 3 to 7 and Figure 1 to 5 the model p-values for all the responses were less than 0.0001, indicating the significance of the model. The coefficient of determination ($R^2$) estimates the proportion of variation in the response that can be attributed to the model rather than to random error. It ranged from 0.9804–0.9999, indicating that the model adequately represented the real relationship between the variables under consideration. The coefficient of variation was within 0.25–1.48, indicating the degree of precision during the biotransformation. It also suggested the high reliability of the experiment. The lack of fit was insignificant, adjusted $R^2$ values, adequate precision values and was given in Table 3 to 7 indicating a good signal-to-noise ratio. The extent of multicollinearity in the statistical analysis of Box-Behnken data is determined by variance inflation factor (VIF). Ideally, it should be close to 1, and values greater than 10 indicate multicollinearity of factors. This occurs if the model comprises factors that are correlated with the response variable as well as to each other. The VIF values for responses were less than 1, indicating that various demographic characteristics and their interactions were predicted effectively without multicollinearity. The interactions of two parameters and their collective effect on the response were studied using contour plots, which are useful for establishing desirable response values and operating conditions. In contour plots, the response surface is viewed as a two-dimensional plane where

all points that have the same response are connected to produce contour lines. If the model contains only the main variables without any interactive effect, the fitted response surface will yield straight lines. The presence of interaction is demonstrated by curved lines. A second-order polynomial is shown having elliptical contours. The responses which show elliptical representation are considered significant, whereas responses that have circular representation are considered negligible. As the level of these parameters changes, their interactive effects on the response also change. The optimization process also gives the effects of significant variables on the responses in the form of an equation. The positive value of coefficients shows a synergistic effect, while a negative value indicates an inverse relation between the variable and the response.

**Table 3: A. Response 1: Apigenin content (mg/ml)**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 21.09 | 9 | 2.34 | 5797.37 | <0.0001 | significant |
| A-Substrate concentration (%) | 0.2664 | 1 | 0.2664 | 659.06 | <0.0001 | |
| B-Inoculum volume (%) | 16.07 | 1 | 16.07 | 39760.12 | <0.0001 | |
| C-Incubation time (hours) | 0.1860 | 1 | 0.1860 | 460.19 | <0.0001 | |
| AB | 0.8836 | 1 | 0.8836 | 2185.58 | <0.0001 | |
| AC | 0.3364 | 1 | 0.3364 | 832.08 | <0.0001 | |
| BC | 0.7569 | 1 | 0.7569 | 1872.19 | <0.0001 | |
| $A^2$ | 1.18 | 1 | 1.18 | 2914.47 | <0.0001 | |
| $B^2$ | 0.6771 | 1 | 0.6771 | 1674.70 | <0.0001 | |
| $C^2$ | 0.7931 | 1 | 0.7931 | 1961.68 | <0.0001 | |
| **Residual** | 0.0028 | 7 | 0.0004 | | | |
| Lack of Fit | 0.0013 | 3 | 0.0004 | 1.22 | 0.4121 | Not significant |
| Pure Error | 0.0015 | 4 | 0.0004 | | | |
| **Cor Total** | 21.10 | 16 | | | | |

## B. Fit Statistics

| | | | |
|---|---|---|---|
| **Std. Dev.** | 0.0201 | **$R^2$** | 0.9999 |
| **Mean** | 7.85 | **Adjusted $R^2$** | 0.9997 |
| **C.V. %** | 0.2560 | **Predicted $R^2$** | 0.9989 |
| | | **Adeq Precision** | 270.4060 |

**Fig: 1 Actual vs Predicted Values Plot for Apigenin Content**

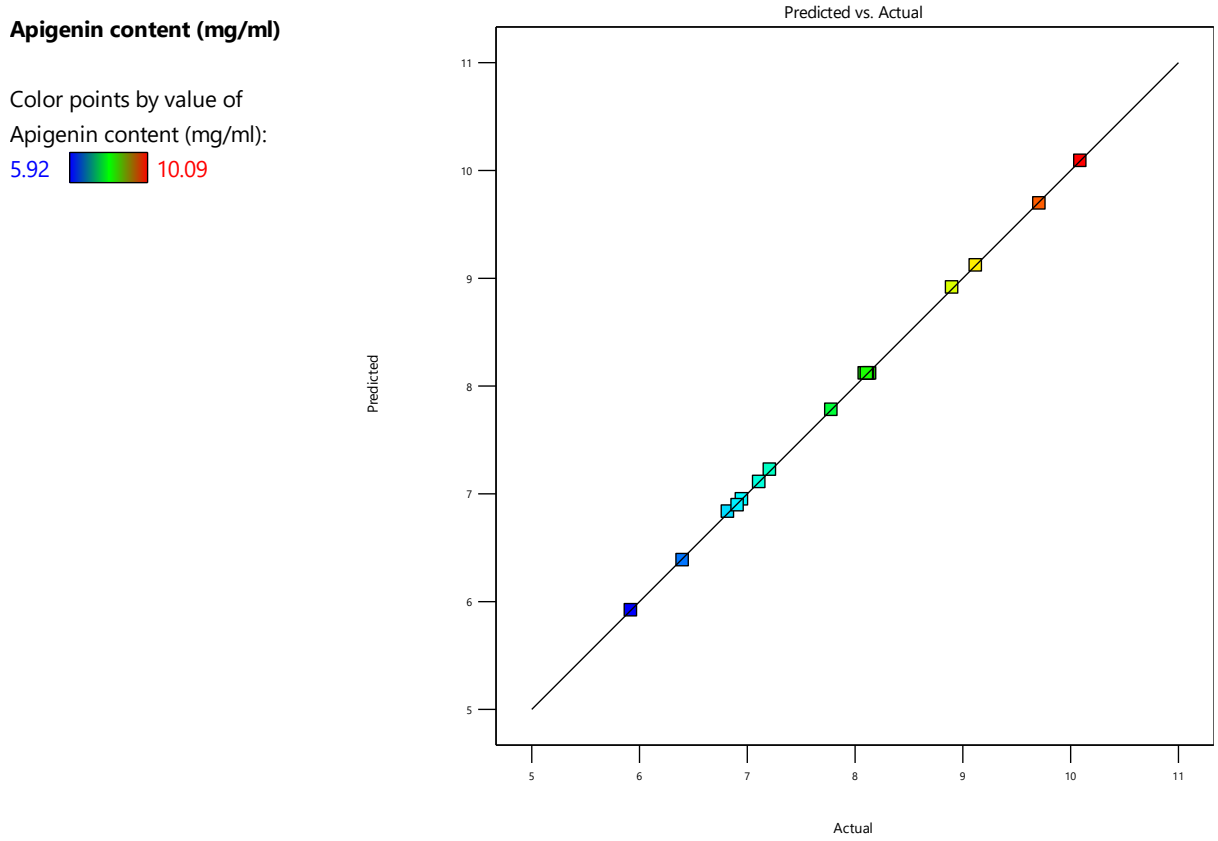


**Table 4: A. Response 2: Luteolin content (mg/ml)**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 5.42 | 9 | 0.6021 | 38.83 | <0.0001 | significant |
| A-Substrate concentration (%) | 0.7626 | 1 | 0.7626 | 49.18 | 0.0002 | |
| B-Inoculum volume (%) | 1.11 | 1 | 1.11 | 71.59 | <0.0001 | |
| C-Incubation time (hours) | 1.30 | 1 | 1.30 | 84.10 | <0.0001 | |
| AB | 0.0812 | 1 | 0.0812 | 5.24 | 0.0559 | |
| AC | 0.5476 | 1 | 0.5476 | 35.31 | 0.0006 | |
| BC | 0.2352 | 1 | 0.2352 | 15.17 | 0.0059 | |
| $A^2$ | 1.17 | 1 | 1.17 | 75.41 | <0.0001 | |
| $B^2$ | 0.1593 | 1 | 0.1593 | 10.27 | 0.0150 | |
| $C^2$ | 0.0061 | 1 | 0.0061 | 0.3921 | 0.5511 | |
| **Residual** | 0.1085 | 7 | 0.0155 | | | |
| Lack of Fit | 0.0362 | 3 | 0.0121 | 0.6679 | 0.6145 | not significant |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pure Error | 0.0723 | 4 | 0.0181 | | | |
| **Cor Total** | 5.53 | 16 | | | | |

## B. Fit Statistics

| | | | |
|---|---|---|---|
| **Std. Dev.** | 0.1245 | **$R^2$** | 0.9804 |
| **Mean** | 8.41 | **Adjusted $R^2$** | 0.9551 |
| **C.V. %** | 1.48 | **Predicted $R^2$** | 0.8747 |
| | | **Adeq Precision** | 20.4044 |

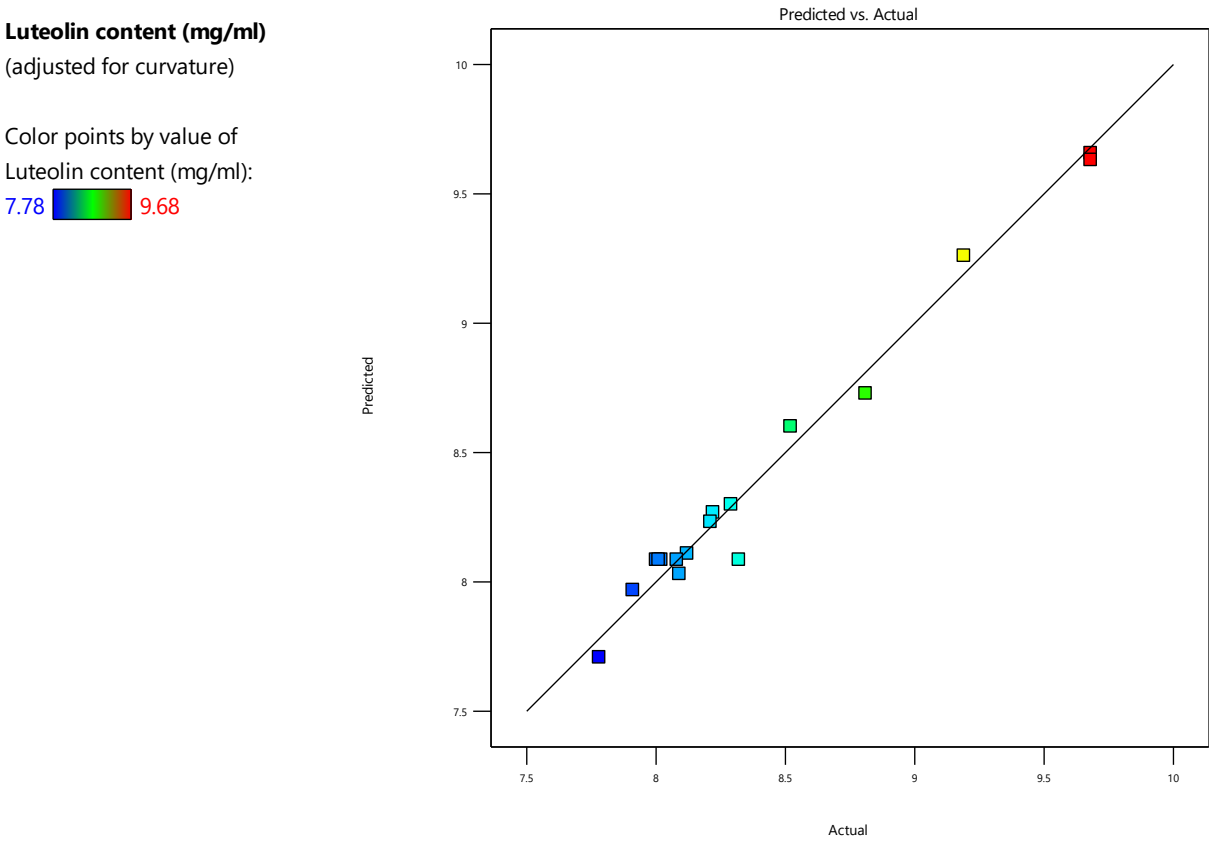


**Fig: 2 Actual vs Predicted Values Plot for Luteolin Content**

**Table 5: A.Response 3: Quercetin content (mg/ml)**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 24.69 | 9 | 2.74 | 133.22 | <0.0001 | significant |
| A-Substrate concentration (%) | 3.51 | 1 | 3.51 | 170.53 | <0.0001 | |
| B-Inoculum volume (%) | 1.07 | 1 | 1.07 | 51.76 | 0.0002 | |
| C-Incubation time (hours) | 18.67 | 1 | 18.67 | 906.56 | <0.0001 | |
| AB | 0.0110 | 1 | 0.0110 | 0.5355 | 0.4881 | |
| AC | 0.2352 | 1 | 0.2352 | 11.42 | 0.0118 | |
| BC | 0.2256 | 1 | 0.2256 | 10.96 | 0.0129 | |
| $A^2$ | 0.4509 | 1 | 0.4509 | 21.90 | 0.0023 | |
| $B^2$ | 0.4105 | 1 | 0.4105 | 19.94 | 0.0029 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| $C^2$ | 0.1048 | 1 | 0.1048 | 5.09 | 0.0587 | |
| **Residual** | 0.1441 | 7 | 0.0206 | | | |
| Lack of Fit | 0.0351 | 3 | 0.0117 | 0.4284 | 0.7439 | not significant |
| Pure Error | 0.1091 | 4 | 0.0273 | | | |
| **Cor Total** | 24.83 | 16 | | | | |

## B. Fit Statistics

| | | | |
|---|---|---|---|
| **Std. Dev.** | 0.1435 | **$R^2$** | 0.9942 |
| **Mean** | 9.77 | **Adjusted $R^2$** | 0.9867 |
| **C.V. %** | 1.47 | **Predicted $R^2$** | 0.9706 |
| | | **Adeq Precision** | 39.7988 |

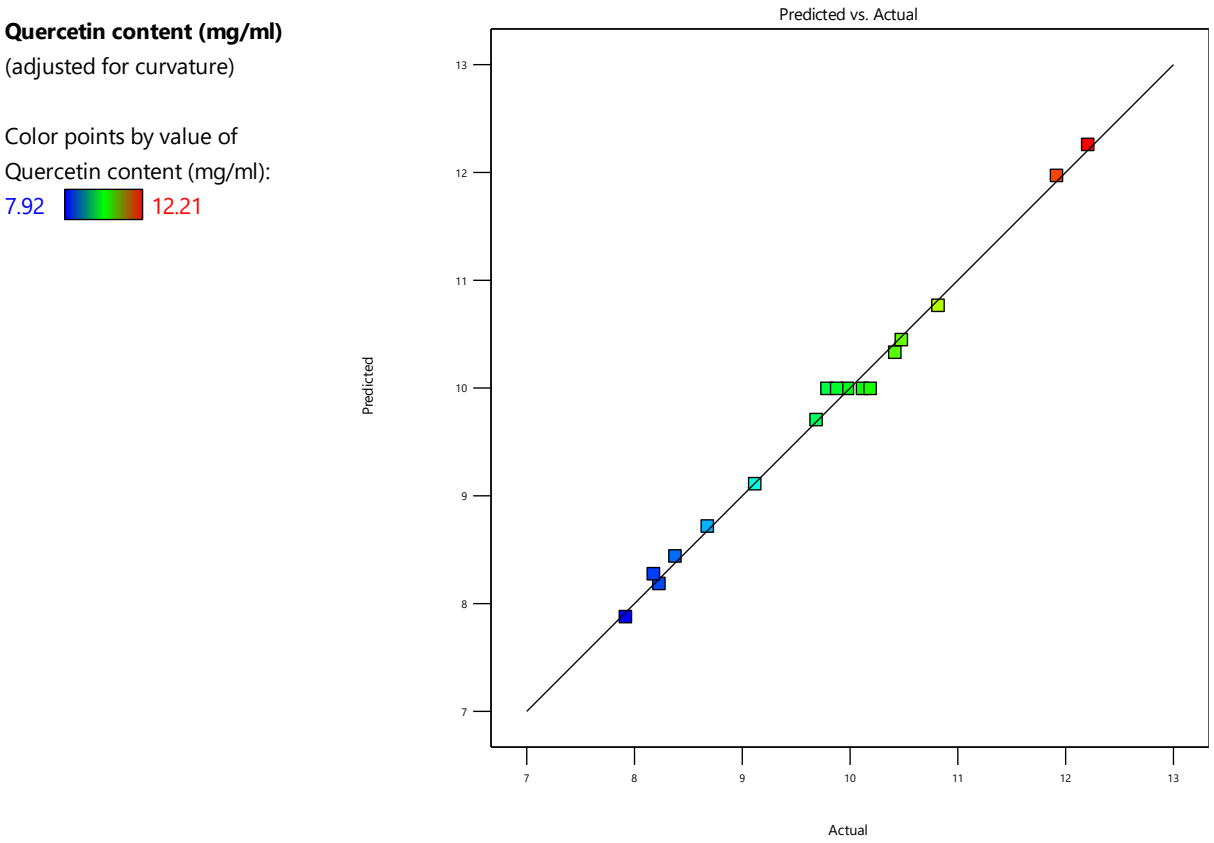


**Fig: 3 Actual vs Predicted Values Plot for Quercetin Content**

### Table 6: A. Response 4: Kaempferol content (mg/ml)

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 20.93 | 9 | 2.33 | 260.81 | <0.0001 | significant |
| A-Substrate concentration (%) | 2.30 | 1 | 2.30 | 257.97 | <0.0001 | |
| B-Inoculum volume (%) | 3.11 | 1 | 3.11 | 349.02 | <0.0001 | |
| C-Incubation time (hours) | 12.15 | 1 | 12.15 | 1362.71 | <0.0001 | |
| AB | 0.0064 | 1 | 0.0064 | 0.7177 | 0.4249 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| AC | 1.48 | 1 | 1.48 | 165.54 | <0.0001 | |
| BC | 1.11 | 1 | 1.11 | 124.81 | <0.0001 | |
| $A^2$ | 0.1327 | 1 | 0.1327 | 14.88 | 0.0062 | |
| $B^2$ | 0.0360 | 1 | 0.0360 | 4.04 | 0.0844 | |
| $C^2$ | 0.5457 | 1 | 0.5457 | 61.19 | 0.0001 | |
| **Residual** | 0.0624 | 7 | 0.0089 | | | |
| Lack of Fit | 0.0078 | 3 | 0.0026 | 0.1911 | 0.8974 | not significant |
| Pure Error | 0.0546 | 4 | 0.0136 | | | |
| **Cor Total** | 21.00 | 16 | | | | |

## B. Fit Statistics

| | | | | |
|---|---|---|---|---|
| **Std. Dev.** | 0.0944 | | **$R^2$** | 0.9970 |
| **Mean** | 9.27 | | **Adjusted $R^2$** | 0.9932 |
| **C.V. %** | 1.02 | | **Predicted $R^2$** | 0.9900 |
| | | | **Adeq Precision** | 53.0700 |

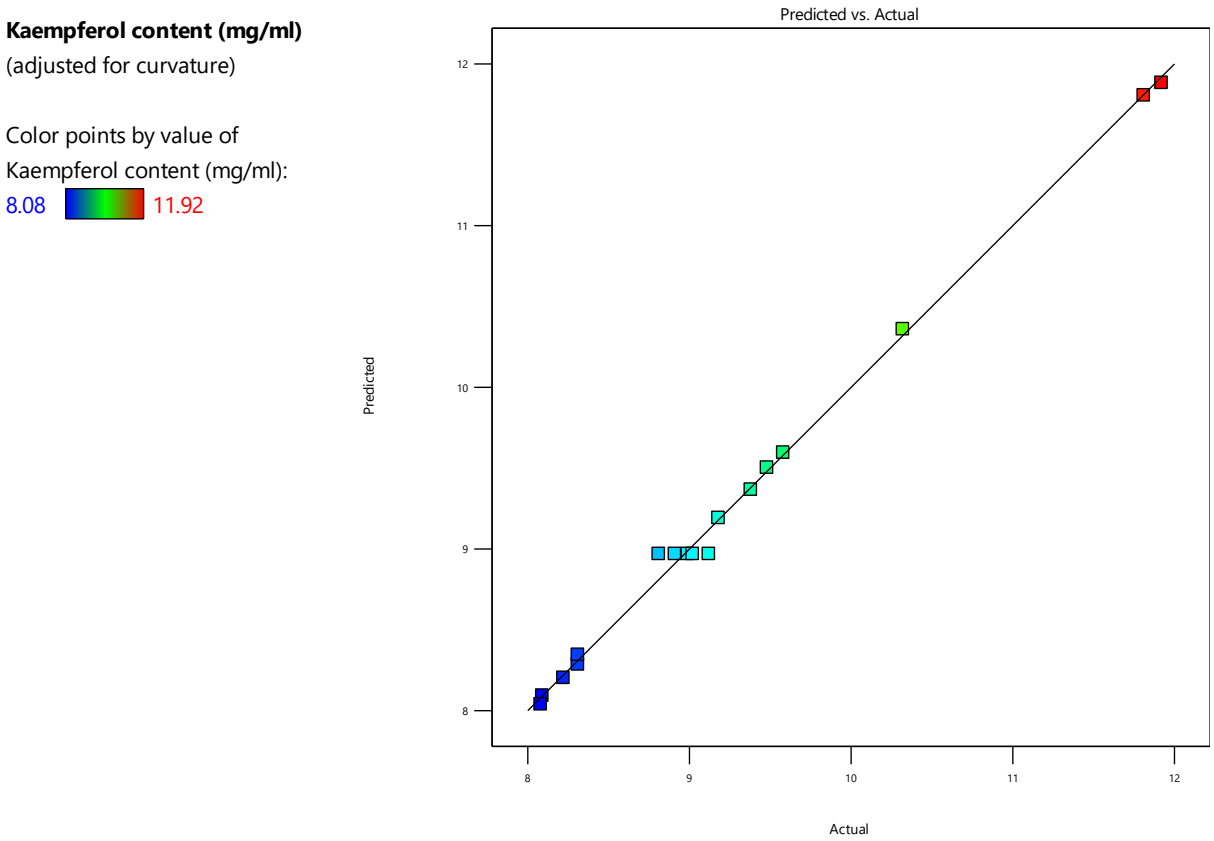


**Fig: 4 Actual vs Predicted Values Plot for Kaempferol Content**

**Table 7: A. Response 5: Isorhamnetin content (mg/ml)**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 6.38 | 9 | 0.7090 | 53.18 | <0.0001 | significant |

| | | | | | | |
|---|---|---|---|---|---|---|
| A-Substrate concentration (%) | 0.8778 | 1 | 0.8778 | 65.84 | <0.0001 | |
| B-Inoculum volume (%) | 0.9045 | 1 | 0.9045 | 67.84 | <0.0001 | |
| C-Incubation time (hours) | 2.04 | 1 | 2.04 | 153.03 | <0.0001 | |
| AB | 0.0812 | 1 | 0.0812 | 6.09 | 0.0429 | |
| AC | 0.7225 | 1 | 0.7225 | 54.19 | 0.0002 | |
| BC | 0.3249 | 1 | 0.3249 | 24.37 | 0.0017 | |
| $A^2$ | 1.26 | 1 | 1.26 | 94.24 | <0.0001 | |
| $B^2$ | 0.0901 | 1 | 0.0901 | 6.76 | 0.0355 | |
| $C^2$ | 0.0171 | 1 | 0.0171 | 1.28 | 0.2946 | |
| **Residual** | 0.0933 | 7 | 0.0133 | | | |
| Lack of Fit | 0.0461 | 3 | 0.0154 | 1.30 | 0.3889 | not significant |
| Pure Error | 0.0472 | 4 | 0.0118 | | | |
| **Cor Total** | 6.47 | 16 | | | | |

## B. Fit Statistics

| | | | |
|---|---|---|---|
| **Std. Dev.** | 0.1155 | **$R^2$** | 0.9856 |
| **Mean** | 8.43 | **Adjusted $R^2$** | 0.9671 |
| **C.V. %** | 1.37 | **Predicted $R^2$** | 0.8746 |
| | | **Adeq Precision** | 25.0402 |

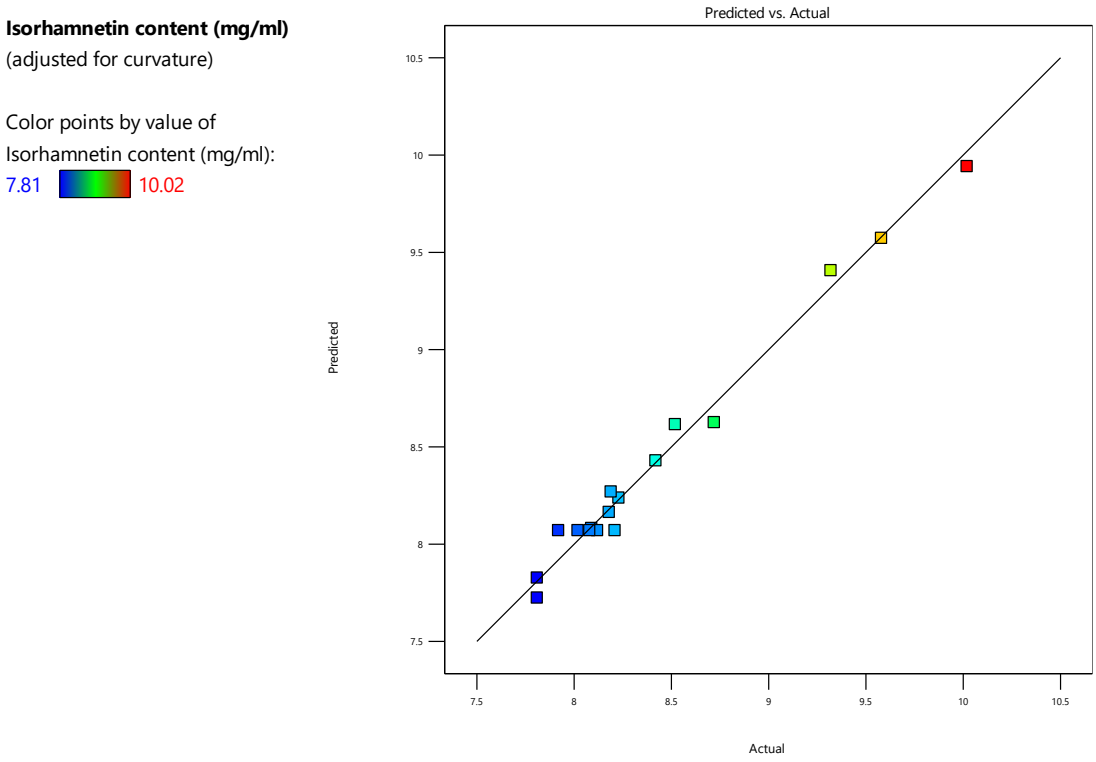


**Fig: 5 Actual vs Predicted Values Plot for Isorhamnetin Content**

## Conclusion

A composite extract comprising *Syzygium cumini* seed, *Mangifera indica* seed, *Momordica charantia* fruit, and *Gymnema sylvestre* was formulated and fermented using *Saccharomyces cerevisiae*. The flavonoid content was analyzed both before and after fermentation, leading to the development of an optimized fermentation method to enhance flavonoid levels. The independent variables optimized were substrate concentration (40%, 60%, and 80%), inoculum volume (2%, 5%, and 8%), and incubation time (40 h, 50 h, and 60 h). The responses measured included the contents of Apigenin, Luteolin, Quercetin, Kaempferol, and Isorhamnetin. A total of 17 experimental runs with 5 center points were predicted by the model. All five responses, showing high predictive accuracy, as the experimental values were closely aligned with the predicted ones. Luteolin, Quercetin, Kaempferol, and Isorhamnetin contents were positively influenced by all three variables, whereas Apigenin content decreased with longer incubation times. Furthermore, the flavonoid levels were affected by interactions among the variables, as illustrated by response surface plots. The maximum yield point was determined using a point prediction tool, and confirmatory experiments validated the model's accuracy. The actual flavonoid content under optimal conditions aligned well with the model's predictions, though the impact on Apigenin content showed a more intricate pattern. Further refinements were achieved through the use of model equations.

**References:**

1. Kumar, S., Saha, S., Singh, K., Singh, T., Mishra, A. K., Dubey, B. N., & Singh, S. (2024). Beneficial effects of spirulina on brain health: A systematic review. *Current Functional Foods*, *3*(1), Article e120124225622. https://doi.org/10.2174/0126668629269256231222092721
2. RaviKKumar VR, Rathi S, Singh S, Patel B, Singh S, Chaturvedi K, Sharma B. A Comprehensive Review on Ulcer and Their Treatment. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2023 Dec 21;39:e20230006. doi: 10.62958/j.cjap.2023.006. PMID: 38755116.
3. Singh, V., Arora, S., Akram, W., Alam, S., Kumari, L., Kumar, N., Kumar, B., Kumar, S., Agrawal, M., Singhal, M., Kumar, S., Singh, S., Singh, K., Saha, S., & Dwivedi, V. (2024). Involvement of molecular mechanism and biological activities of Pemirolast: A therapeutic review. *New Emirates Medical Journal*, *5*, Article e02506882308410. https://doi.org/10.2174/0102506882308410240607053814
4. Rajput DS, Gupta N, Singh S, Sharma B. A Comprehensive Review: Personalized Medicine for Rare Disease Cancer Treatment. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2023 Dec 23;39:e20230008. doi: 10.62958/j.cjap.2023.008. PMID: 38830754.
5. Singh S, Chaurasia A, Rajput DS, Gupta N. Mucoadhesive Drug Delivery System and There Future Prospective: Are a Promising Approach for Effective Treatment? Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2023 Dec 20;39:e20230005. doi: 10.62958/j.cjap.2023.005. PMID: 38751344.
6. Kumar, S., Saha, S., Sharma, B., Singh, S., Shukla, P., Mukherjee, S., Agrawal, M., Singh, K., & Singh, T. (2023). The role of resveratrol in Alzheimer's disease: A comprehensive review of current research. *Current Functional Foods*, *2*(2), Article e121223224364, 13 pages. https://doi.org/10.2174/0126668629269244231127071411
7. Patel S, Ismail Y, Singh S, Rathi S, Shakya S, Patil SS, Bumrela S, Jain PC, Goswami P, Singh S. Recent Innovations and Future Perspectives in Transferosomes for Transdermal Drug Delivery in Therapeutic and Pharmacological Applications. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2024 Oct 24;40:e20240031. doi: 10.62958/j.cjap.2024.031. PMID: 39442957.

8. Vaghela MC, Rathi S, Shirole RL, Verma J, Shaheen, Panigrahi S, Singh S. Leveraging AI and Machine Learning in Six-Sigma Documentation for Pharmaceutical Quality Assurance. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2024 Jul 18;40:e20240005. doi: 10.62958/j.cjap.2024.005. PMID: 39019923.
9. Patel S, Ismail Y, Singh S, Rathi S, Shakya S, Patil SS, Bumrela S, Jain PC, Goswami P, Singh S. Recent Innovations and Future Perspectives in Transferosomes for Transdermal Drug Delivery in Therapeutic and Pharmacological Applications. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2024 Oct 24;40:e20240031. doi: 10.62958/j.cjap.2024.031. PMID: 39442957.
10. Kumar, S., Saha, S., Pathak, D., Singh, T., Kumar, A., Singh, K., Mishra, A. K., Singh, S., & Singh, S. (2024). Cholesterol absorption inhibition by some nutraceuticals. *Recent Advances in Food, Nutrition & Agriculture*, *16*(1), 2–11. https://doi.org/10.2174/012772574X285280240220065812
11. Singh, S., Chaurasia, A., Rajput, D. S., & Gupta, N. (2024). An overview on mucoadhesive buccal drug delivery systems & approaches: A comprehensive review. *African Journal of Biological Sciences (South Africa)*, *6*(5), 522–541, DOI: 10.33472/AFJBS.6.5.2024.522-541
12. Kumar, S., Singh, S., Rajput, D., Sharma, B., Chaturvedi, K., Singh, N., Saha, S., Singh, K., & Mukherjee, S. (2024). Pharmacological approaches and herbal interventions for Alzheimer's disease. *The Natural Products Journal*, *14*(8), Article e220124225945. https://doi.org/10.2174/0122103155275266231123090138
13. Ravikkumar VR, Patel BD, Rathi S, Parthiban S, Upadhye MC, Shah AM, Rehan SSA, Samanta S, Singh S. Formulation and Evaluation of Drumstick Leaves Tablet as An Immunomodulator. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2024 Jun 21;40:e20240004. doi: 10.62958/j.cjap.2024.004. PMID: 38902996.
14. Sharma, A., Bara, G., Keshamma, E., Sharma, B., Singh, S., Singh, S. P., Parashar, T., Rathore, H. S., Sarma, S. K., & Rawat, S. (2023). Cancer biology and therapeutics: A contemporary review. *Journal of Cardiovascular Disease Research*, *14*(10), 1229-1247.
15. Dewangan, H. K., Singh, S., Mishra, R., & Dubey, R. K. (2020). A review on application of nanoadjuvant as delivery system. *International Journal of Applied Pharmaceutics*, *12*(4), 24–33. https://doi.org/10.22159/ijap.2020v12i4.36856
16. Singh S, Chaurasia A, Gupta N, Rajput DS. Effect of Formulation Parameters on Enalapril Maleate Mucoadhesive Buccal Tablet Using Quality by Design (QbD) Approach. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2024 Jun 27;40:e20240003. doi: 10.62958/j.cjap.2024.003. PMID: 38925868.