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# Optimization of Catechin-Mediated Inhibition of α-Amylase and α-Glucosidase Using Response Surface Methodology: Implications for Glycemic Control.

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## Abstract

**Background:**

Managing postprandial hyperglycemia is an important target for the treatment of type 2 diabetes mellitus (t2dm). One strategy is to reduce the activity of enzymes responsible for carbohydrate digestion, such as α-amylase and α-glucosidase. In recent years, natural polyphenols like catechin and epicatechin have been studied for their potential metabolic effects. Experimental data suggest potential impacts on glucose metabolism and insulin sensitivity; however, evidence from integrated human models is limited and needs further clarification.

**Objective:**

this study examined the effects of catechin and epicatechin supplementation on digestive enzyme activity and glycemic control in individuals with type 2 diabetes, using response surface methodology to analyze metabolic interactions.

**Methods:**

in a central composite design study, 192 participants (diabetic and healthy) received 200 mg/day of catechins (100 mg catechin and 100 mg epicatechin twice daily) for 45 days. Fasting serum samples were analyzed for α-amylase, α-glucosidase, and β-glucosidase activities, as well as glucose and insulin levels. Serum underwent dialysis and sequential molecular weight filtration before rp-hplc separation on a c18 column. Enzyme activity was determined spectrophotometrically, and insulin concentrations by elisa. A desirability function, based on the geometric mean of individual outcomes, summarized the overall metabolic response. Anova and goodness-of-fit indicators assessed model performance.

**Results:**

after supplementation, the activities of α-amylase, α-glucosidase and β-glucosidase were significantly reduced ($p < 0.005$). These enzymatic changes were accompanied by lower serum glucose values and an increase in circulating insulin levels. The statistical model demonstrated high explanatory power ($r^2 = 95.66\%$; adjusted $r^2 = 95.29\%$) without significant lack-of-fit. Age and bmi were found to play a role in response magnitude. The improvement was more pronounced in younger participants and in those with lower bmi. Interestingly, nearly half (45%) of diabetic subjects receiving supplementation exhibited response patterns comparable to those of healthy individuals according to the integrated index.

**Conclusion:**

the results suggest that daily catechin and epicatechin (200 mg/day) intake may assist with better glycemic regulation, as evidenced through possibly partial inhibition of carbohydrate-digesting enzymes and modulation of insulin dynamics. Such variation of response between age and bmi categories indicates that patient characteristics should be taken into account when assessing flavonoids in clinical interventions. More longitudinal and larger studies are required to verify these observations.

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**Keywords:** type 2 diabetes; catechin; epicatechin; α-amylase; α-glucosidase; insulin; response surface methodology; human study.

## Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from defects in insulin secretion, insulin action, or both. It represents a major global health concern, with its prevalence increasing substantially over recent decades and contributing significantly to global morbidity and mortality (International Diabetes Federation **[IDF], 2023;** World Health Organization **[WHO], 2023).** Poor glycemic control is strongly associated with long-term microvascular and macrovascular complications, underscoring the importance of effective strategies to regulate postprandial blood glucose levels (American Diabetes Association **[ADA], 2024).**

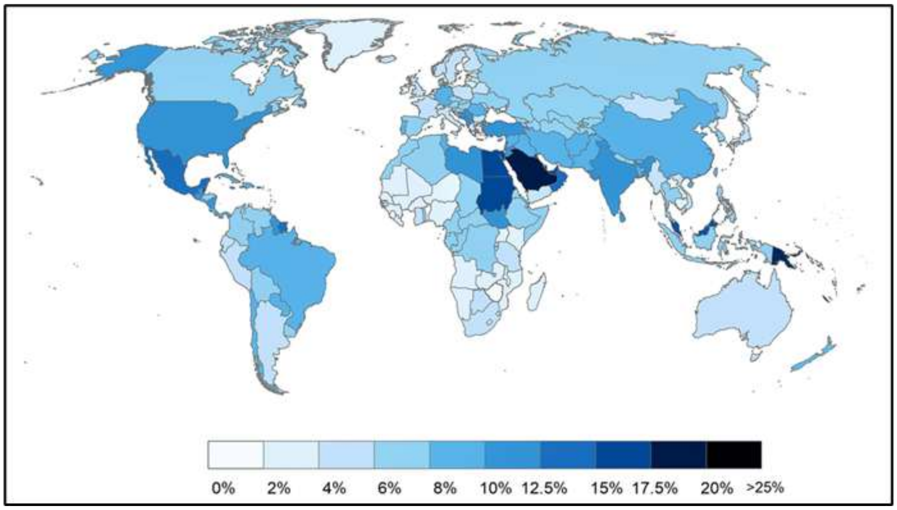


**figure 1-1) spread of diabetes until 2020**

One of the established therapeutic strategies for controlling postprandial hyperglycemia involves the inhibition of key carbohydrate-digesting enzymes, namely α-amylase and α-glucosidase. These enzymes are responsible for the hydrolysis of complex carbohydrates into absorbable monosaccharides. Their inhibition delays carbohydrate digestion and glucose absorption, thereby reducing postprandial glucose excursions **(Kumar et al., 2021; Sales et al., 2022).** Although synthetic inhibitors such as acarbose are widely used, their administration is often associated with gastrointestinal side effects, prompting the exploration of safer natural alternatives (Lebovitz, 2021).Catechins, a group of polyphenolic flavonoids abundantly found in tea and various medicinal plants, have gained considerable attention due to their antioxidant, anti-inflammatory, and antidiabetic properties **(Shay et al., 2020; Zhang et al., 2022)**.

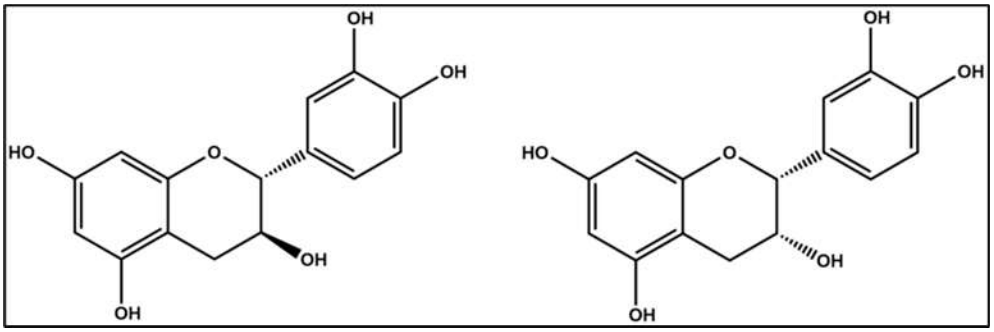


**figure 1-2 : chemical structures of catechin and epicatechin**

Several in vitro investigations have demonstrated that catechins exhibit inhibitory activity against α-amylase and α-glucosidase through potential interactions with catalytic residues at the enzyme active sites (**Gao et al., 2021; Li et al., 2023**). These inhibitory effects are believed to involve hydrogen bonding and hydrophobic interactions mediated by hydroxyl groups within the catechin molecular structure **(Xu et al., 2022).** However, inhibitory efficiency may vary depending on concentration, environmental conditions, and interaction dynamics.

Despite the expanding literature on catechin-mediated enzyme inhibition, limited studies have systematically optimized inhibitory conditions using advanced statistical modeling approaches. Conventional experimental designs frequently rely on single-variable analysis, which does not sufficiently account for potential interaction effects between variables **(Montgomery, 2019).** Response Surface Methodology (RSM) is a robust statistical and mathematical technique that enables simultaneous evaluation of multiple factors and their interactions, allowing precise optimization of experimental conditions **(Bezerra et al., 2020; Myers et al., 2016)**. The application of RSM in enzyme inhibition research enhances predictive accuracy and experimental efficiency compared to traditional approaches.

Therefore, the present study aimed to optimize catechin-mediated inhibition of α-amylase and α-glucosidase using Response Surface Methodology. By integrating biochemical evaluation with statistical modeling, this study seeks to enhance inhibitory efficiency and provide scientific insight into the potential application of catechins as natural agents for glycemic control.

## Materials and methods

Catechin, epicatechin, insulin, glucose, α-amylase, α-glucosidase, β-glucosidase, bovine serum albumin (BSA), $Na_2HPO_4$, and $NaH_2PO_4$ were obtained from Sigma-Aldrich (Germany). Methanol, acetonitrile, and HPLC-grade water were purchased from Merck (Germany). All reagents were of analytical grade and used without further purification.

## Human Subjects

Human samples were collected in collaboration with the Iran Diabetes Center and Iran Diabetes Association. The study design was generated using Design-Expert software based on the predictive algorithm described by Al-Baidhani et al. (2022).

Participants received oral supplementation of catechin and epicatechin (100 mg/day) for 45 days. Blood samples (10 mL) were collected under approved clinical ethics protocol (ISBT402-0032801CR) at Mustafa Khomeini Hospital.

Serum samples were separated by centrifugation and anonymized before analysis.

Instrumentation

UV–Visible Spectrophotometry

Enzyme activity measurements were performed using a PowerWave XS2 UV–Vis microplate reader (BioTek, USA), equipped with temperature control (25–60°C) and multi-wavelength scanning (200–800 nm).

High-Performance Liquid Chromatography (HPLC)

HPLC analysis was conducted using a Knauer system (Berlin, Germany) equipped with a K-1001 pump and K-2800 UV detector.

Separation was performed on a Zorbax 300SB-C18 column (4.6 × 250 mm, 5 µm).

Mobile phases:

- A: Water/acetonitrile (95:5) + 1% TFA
- B: Water/acetonitrile (5:95) + 1% TFA

Gradient:

5–50% B (0–30 min), then 50–95% B (30–60 min).

Detection was performed at the appropriate wavelength.

Calibration Procedures

Protein Calibration

BSA standards (10–200 µg/mL) were used to construct a calibration curve. Linear regression yielded:

$Y=3811.1X-24495$

$R^2 = 0.9968$

Glucose Calibration

Glucose standards (5–1000 µg/mL) were prepared using the phenol–sulfuric acid method. Linear regression:

$Y=0.00021X+0.66$

$R^2 = 0.9923$

Enzyme Activity Assays

α-Amylase Inhibition Assay

The assay was performed using the DNS method at pH 6.9 and 20°C.

Reaction mixture contained:

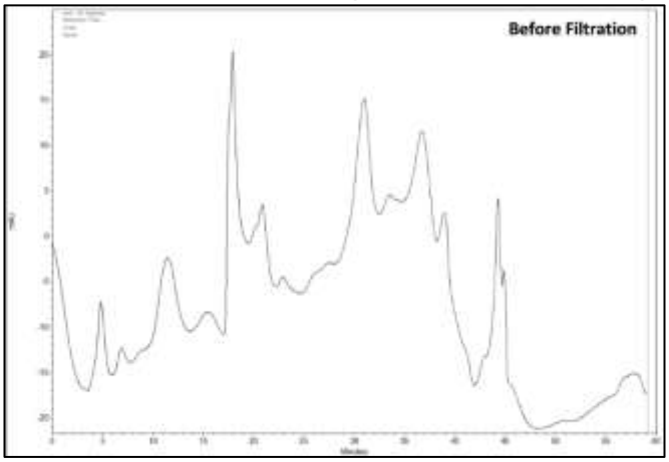
- 200 µL starch solution (1%)
- 100 µL sample
- 200 µL α-amylase (2 mg/mL)

After incubation, DNS reagent was added and heated at 100°C for 15 min. Absorbance was measured at 540 nm.
Inhibition (%) was calculated as:
Inhibition%=[(A_control − A_sample) / A_control] × 100
All experiments were conducted in triplicate.

α-Glucosidase Inhibition Assay
The assay was performed using PNPG substrate at 37°C.
Reaction mixture contained

- 40 µL sample
- 80 µL enzyme (0.5 U/mL)
- 80 µL PNPG
- 320 µL sodium carbonate (stop solution)

Absorbance was recorded at 405 nm.
Inhibition percentage was calculated using the same formula described above.



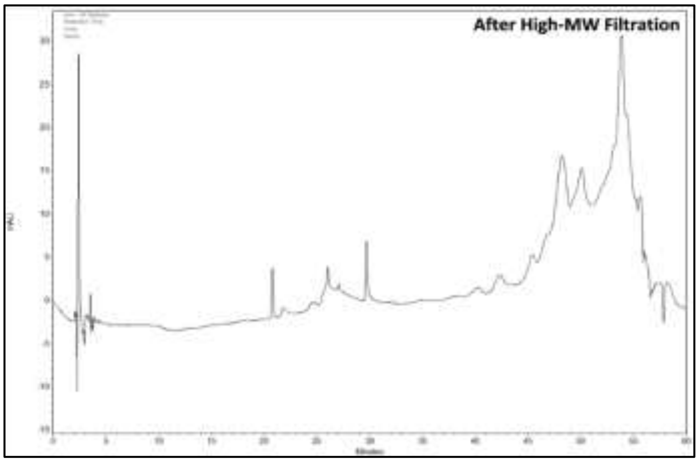


**figure 2-3-2 chromatogram after 100 kda filtration figure2-3-3: chromatogram after 40 kda filtration**

Rp-hplc analysis was performed using a c18 column (4.6 × 250 mm, 5 µm, 300 å). The mobile phase consisted of:

- Phase a: 5% acetonitrile (1% tfa)
- Phase b: 95% acetonitrile (1% tfa)

A linear gradient (5–50% b, 30 min; 50–95% b, 30 min) was applied.

2.4 biochemical assays

*A-amylase inhibition*

Measured spectrophotometrically at 540 nm using starch/dns method.
Inhibition (%) = (a_control − a_sample) / a_control × 100

*A-glucosidase and β-glucosidase*

Measured at 405 nm using pnpg substrate.

***Serum glucose***

Determined using phenol–sulfuric acid assay.
Insulin quantified using sandwich elisa at 450 nm.

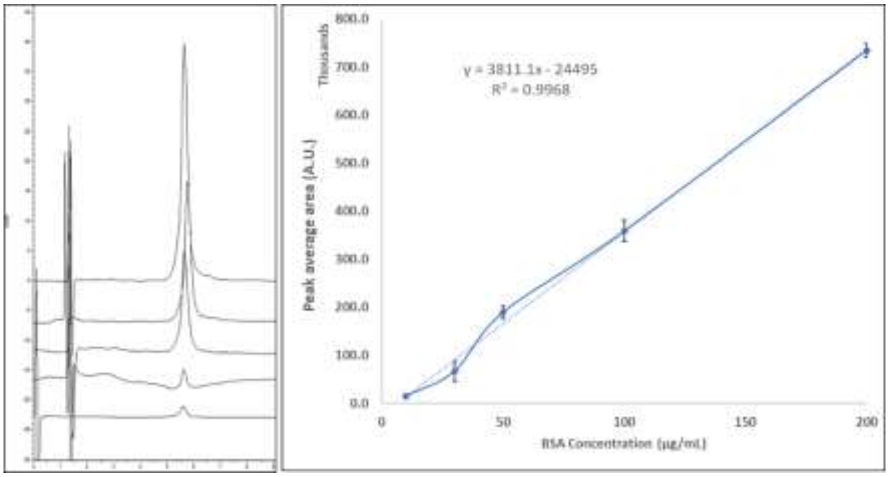


**figure 2-3-4 glucose calibration curve**

## 2.5 statistical modeling and desirability function

Anova and response surface modeling were performed using design-expert software.

To integrate the five metabolic responses, an overall desirability index (d) was calculated as the geometric mean of individual desirability functions:

D=(d1×d2×d3×d4×d5)1/5d = (d_1 \times d_2 \times d_3 \times d_4 \times d_5)^{1/5}d=(d1×d2×d3×d4×d5)1/5

Model adequacy was evaluated using:

- Coefficient of determination ($r^2$)
- Adjusted $r^2$
- Lack-of-fit test
- Model f-value

Statistical significance was set at $p < 0.05$.

3.1 model adequacy and multivariate statistical performance

A total of 192 experimental observations generated through the central composite design matrix were included in the response surface analysis. The integrated desirability index (d), representing the geometric aggregation of enzyme inhibition (α-amylase, α-glucosidase, β-glucosidase), serum glucose reduction, and insulin elevation, was defined as the primary response variable.

Anova confirmed that the quadratic response surface model was highly significant ($p < 0.005$). The model demonstrated strong explanatory and predictive capability ($r^2 = 0.9566$; adjusted $r^2 = 0.9529$), indicating that more than 95% of the variability in the integrated metabolic response was explained by the included factors. The lack-of-fit test was non-significant ($p > 0.05$), confirming model adequacy and absence of systematic deviation.

All five independent variables—age (a), bmi (b), physical activity (c), health status (d), and supplement intake (e)—exerted statistically significant main effects on the integrated desirability response ($p < 0.05$). Interaction between health status and supplementation (d×e) of two factors exhibited the highest f-value, indicating that the metabolic response to flavan-3-ol supplementation was strongly dependent on baseline glycemic status.

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### 3.2 effect of catechin and epicatechin supplementation on metabolic endpoints

Combined supplementation (200 mg/day) resulted in significant inhibition of carbohydrate-digesting enzymes. Compared with non-supplemented groups, supplemented individuals demonstrated:

- Reduced α-amylase activity
- Reduced α-glucosidase activity
- Reduced β-glucosidase activity
- Decreased serum glucose levels
- Increased circulating insulin concentration

All changes were statistically significant ($p < 0.005$).

The integrated desirability index revealed a distinct separation between healthy people and diabetics. Notably, approximately 45% of diabetic subjects receiving supplementation achieved d-values within the reference range observed in healthy controls, indicating substantial metabolic normalization in a clinically relevant subgroup.

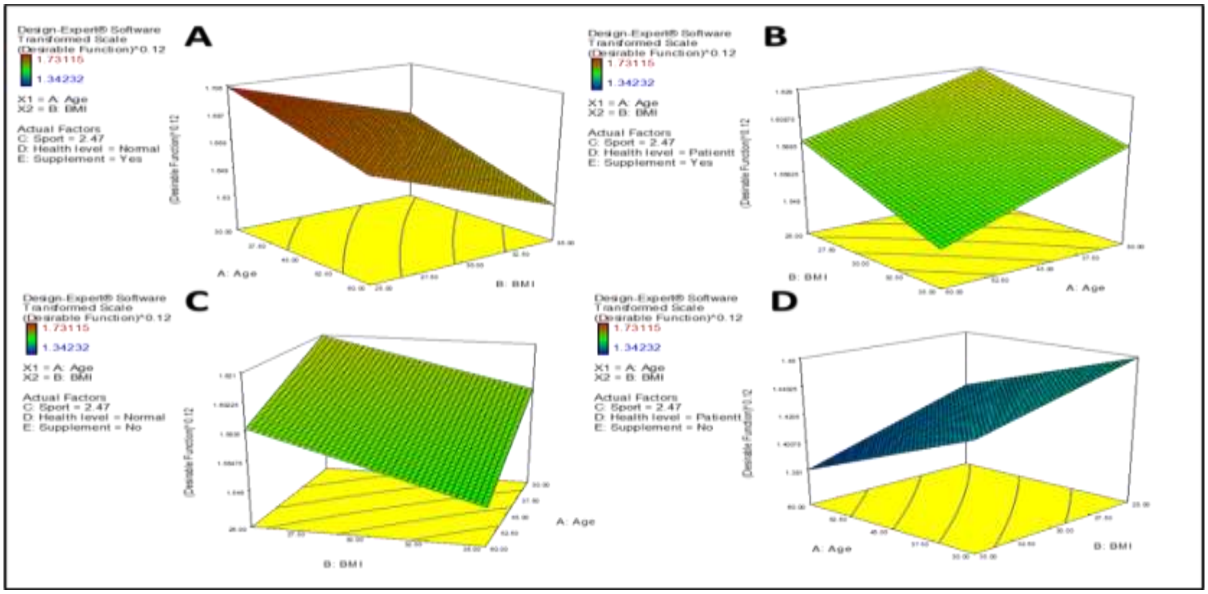

**Figure 3-2-1-1glucose index is related to people with old age and low sports activity age and bmi on df response.**

### 3.3 response surface and interaction analysis

#### *3.3.1 age × bmi interaction (a×b)*

Three-dimensional response surface plots showed the interaction of age with bmi to be prominent. Supplement efficacy decreased with increasing age and bmi. For younger participants with low bmi the highest d-values represented maximal metabolic outcomes. Conversely elderly subjects with high bmi presented decreased enzymatic inhibition, and less reduction in serum glucose. This interaction implies a synergistic adverse effect of age-related metabolic decline and adiposity-driven insulin resistance on flavan-3-ol responsiveness.

***3.3.2 age × physical activity interaction (a×c)***

Moderate physical activity (≥3 sessions/week) significantly enhanced supplementation efficacy, especially among younger individuals. The response surface indicated that physical activity partially mitigated age-associated decline in metabolic responsiveness, suggesting an interaction between lifestyle factors and polyphenol-mediated enzyme modulation.

***3.3.3 bmi × physical activity interaction (b×c)***

In healthy individuals, lower BMI combined with moderate physical activity yielded the highest integrated metabolic optimization. In diabetic participants receiving supplementation, BMI exerted a stronger influence than physical activity, indicating that adiposity is a dominant determinant of enzymatic and glycemic response.

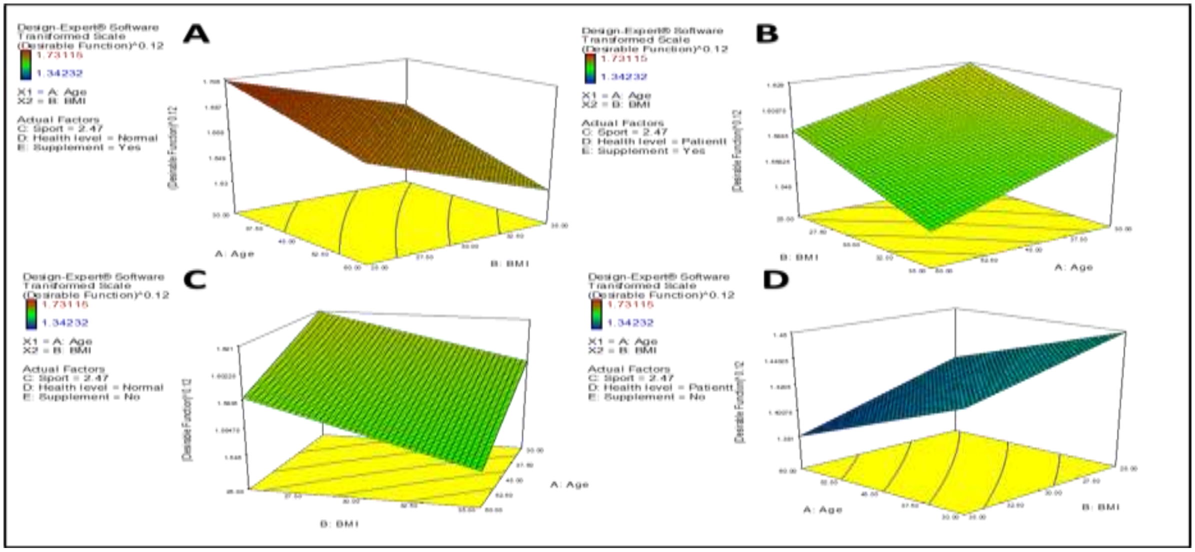

**Figure 3-2-2 response surface produced based on the designed model according to the results of df human study. The effect of two simultaneous factors of age and amount of sports activity (ac) in healthy people receiving supplements: (a) sick people receiving supplements (b), healthy people not receiving supplements (c) and sick people not receiving supplements.**

## Results and Discussion

4.1 Chemicals and Reagents

Catechin, epicatechin, insulin, glucose, α-amylase, α-glucosidase, β-glucosidase, bovine serum albumin (BSA), $Na_2HPO_4$, and $NaH_2PO_4$ were obtained from Sigma-Aldrich (Germany). Methanol, acetonitrile, and HPLC-grade water were purchased from Merck (Germany). All chemicals were of analytical grade and used without further purification.

4.2 Enzyme Inhibition Assay

4.2.1 α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined using the 3,5-dinitrosalicylic acid (DNS) method. The reaction mixture consisted of 200 µL of 1% starch solution, 100 µL of sample, and 200 µL of α-amylase solution (2 mg/mL) prepared in 20 mM phosphate buffer (pH 6.9).

After incubation at 20°C for 3 minutes, 200 µL of DNS reagent was added, and the mixture was heated at 100°C for 15 minutes. Following cooling, absorbance was measured at 540 nm using a UV–Vis microplate reader.

Enzyme inhibition (%) was calculated as:

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

All experiments were performed in triplicate.

### 4.2.2 α-Glucosidase Inhibition Assay

The α-glucosidase inhibitory activity was measured using p-nitrophenyl-α-D-glucopyranoside (pNPG) as substrate. The reaction mixture contained 40 µL sample, 80 µL enzyme solution (0.5 U/mL), and phosphate buffer. After incubation at 37°C for 15 minutes, 80 µL pNPG was added and incubated for an additional 15 minutes.

The reaction was terminated by adding 320 µL sodium carbonate solution. Absorbance was recorded at 405 nm.

Inhibition percentage was calculated using the same formula described above.

## 4.3 Experimental Design and RSM Modeling

Response Surface Methodology (RSM) was employed to optimize the inhibitory activity of catechin against α-amylase and α-glucosidase. The experimental design was generated using Design-Expert software (version X).

Independent variables included catechin concentration, incubation time, and temperature. A second-order polynomial model was applied to describe the relationship between independent variables and enzyme inhibition response.

Model adequacy was evaluated using analysis of variance (ANOVA), coefficient of determination ($R^2$), and lack-of-fit testing.

## 4.4 Statistical Analysis

All experiments were conducted in triplicate, and results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Design-Expert and SPSS software. A $p$-value $< 0.05$ was considered statistically significant (Fig:4-1)

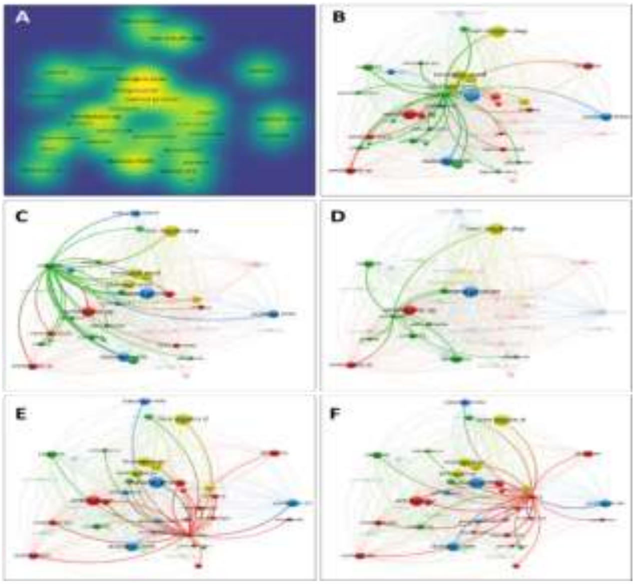

**Figure 4-1 analysis of information sources based on cvs-scopus algorithm using vosviewer software (initial result in figure 1-3) for research keywords glucose (a), amylase enzyme (b), glucosidase (c and d), insulin (e) and catechin (f), with 99.5% accuracy in 188,904 related information sources from 2000 to 2024.**

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