"QUALITY BY DESIGN APPROACH TO FORMULATE AN ANTI-DIABETIC HERBAL DRUG PRODUCT"

Thesis submitted to

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For the award of the degree of

Doctor of Philosophy
In the Faculty of Pharmacy

By

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(Registration No: KLEU/Ph.D./18-19/DO1218012)

Under the Guidance of

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Vishakha M. Parab Gaonkar

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LIST OF ABBREVATIONS

AQbD: Analytical Quality by Design

ANOVA: Analysis of variance

ATP: Analytical Target Profile

AUC: Area under the curve

BDC: Bis-demethoxycurcumin

CCS: Crosscarmellose sodium

CFU: Colony forming unit

CQA: Critical Quality Attributes

CUR: Curcumin

DoE: Design of Experiments

DM: Diabetes Mellitus

DMC: Demethoxycurcumin

FTIR: Fourier-transform infrared

GYM: Gymnemagenin

HDL: High-density lipoprotein

HPLC: High Performance Liquid Chromatography

ICH: International Council for Harmonization

IC_{50}: Half Maximal inhibitory concentration

LDL: Low density lipoprotein

LOD: Limit of detection

LOQ: Limit of Quantification

MCC: Microcrystalline cellulose

MODR: Method Operable Design Region

min: minute

mm: millimeter

mg: milligram

ml: milliliter

NLT: Not less than

NMT: Not more than

nm: nanometer

OF: Optimized Formulation

OGTT: Oral Glucose Tolerance Test

OPA: Ortho-phosphoric acid

PRN: Piperine

QbD: Quality by Design

QTPP: Quality Target Product Profile

RA: Risk assessment

RH: Relative humidity

RP-HPLC: Reversed Phase High Performance Liquid Chromatography

RSD: Relative standard deviation

STZ: Streptozocin

SD: Standard deviation

SEM: Standard error of mean

TG: Triglyceride

TC: Total cholesterol

TRG: Trigonelline

VLDL: Very Low density lipoprotein

µg: microgram

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Abstract

Background: Regardless of great advances in modern system of medicine, traditional medicine is still considered as the primary form of treating diseases in majority the countries. However, the lack of standard quality control profiles is the major focus of attention for the herbal medicines. Hence, Quality by Design (QbD) concept can serve as novel approach to overcome the problems related to herbal drug development.

Objectives: the objective of the present research work is;

1. To establish a Quality protocol for development of herbal drug product by application of Quality by Design (QbD) Approach.
2. Formulation and evaluation of an anti-diabetic herbal drug product with respect to above developed Quality Protocol.

Methodology: the herbal raw materials namely, Gymnema sylvestre, Trigonella foenum-graecum, Curcuma longa and Piper nigrum were selected for the development of anti-diabetic formulation. The selected four herbal raw materials were then subjected for quality assessment. Analytical QbD assisted HPLC method was developed for the quantification of phytocompounds in selected herbs. Further, each selected herbal raw materials was subjected to extraction and the extracts from each herbal raw material was evaluated for various quality evaluation parameters. Formulation of herbal anti-diabetic tablets was executed with the help of QbD paradigm. Initially QTPP was developed followed by identification of CQAs. The risk assessment for the formulation of herbal tablets was conducted by using an Ishikawa (Fish-bone) diagram and a relative risk-ranking system. A 3^2 factorial design was utilized for designing the formulation trials. Further, the design space was established by setting the targeted ranges for each CQA

and optimized formulation batch was identified. Further, the optimized formulation was subjected for stability studies. Evaluation of anti-diabetic activity of the optimized tablet batch was evaluated by in-vitro enzyme inhibition assays and in-vivo animal activity in streptozocin induced diabetic rat model.

Results: the herbal raw materials demonstrated the results within the given standard limits for all the quality evaluation parameters studied. The quantitative estimation revealed the presence of 5.11, 0.58, 9.32, 10.98, 13.86, and 3.63 %w/w of Gymnemagenin, Trigonelline, bis-demethoxy Curcumin, Demethoxycurcumin, Curcumin and Piperine in the raw herbal samples. For the QbD assisted formulation development, disintegration time (Y1), hardness (Y2) and friability (Y3) were identified as CQAs. Concentration of MCC (X1) and CCS (X2) was selected as independent variation for execution of DoE. A total 9 formulation batches were executed and formulation batch F-3 was predicted to be optimized one from the design space. The stability study results indicated that herbal anti-diabetic tablets were stable when exposed to different temperature conditions for 3 months. The *in-vitro* α-glucosidase inhibitory activity reflected the IC50 value 291.70 µg/ml by the formulation and 70.95µg/ml by acarbose. Similarly, the IC50for the inhibition of α-amylase was found to be 572µg/ml by the formulation and 61.32µg/ml by acarbose. *In-vivo* animal activity revealed the decrease in blood glucose level, total cholesterol, triglycerides, LDL and VLDL in diabetic rats treated with optimized formulation F-3.

Conclusion: The present research work demonstrates a comprehensive QbD approach for the systematic design and development of herbal drug products. The current study will

assist readers in understanding the nitty-gritty of applying the QbD concept for the development of herbal formulations.

Key words: Quality by Design, Anti-diabetic, *Gymnema sylvestre*, *Trigonella foenum-graecum*, *Curcuma longa*, *Piper nigrum*

1. INTRODUCTION

1.1. BACKGROUND

Ayurveda, a term meaning Ayur (life) and Veda (knowledge) is derived from the Sanskrit language. It is regarded as one of the oldest systems of medicine, having been practiced since ancient times prior to the establishment and spread of modern medicine and continuing to be used today. As a source of treatment, ayurvedic medicines include medicinal herbs, minerals, metal compounds, etc.[1] Amongst these, herbal medicines are frequently utilized to treat a wide range of ailments. Herbal medicines are plant-based materials with therapeutic or beneficial effects on human health, containing raw and processed parts of one or more plants.[2]

Diabetes mellitus (DM) is one of the most prevalent diseases in all parts of the world and a severe threat to human health. According to WHO, "Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose, which leads over time to serious damage to the heart, blood vessels, eyes, kidneys, and nerves".[3] It is a complicated, diverse group of metabolic disorders that includes hyperglycemia and is linked to carbohydrate, protein, and lipid metabolism imbalances.[4]According to recent data from the International Diabetes Federation (IDF), roughly 463 million persons are currently living with diabetes, with predictions that this number will rise to 578 million by 2030 and 700 million by 2045.[5]

The management of DM has become a global concern. Currently, insulin and oral hypoglycemic agents are considered as the primary therapeutic options for the control and management of diabetes by the allopathic system of medicine. Nevertheless, due to complications associated with them, poor tolerability, high costs, and other side effects

limit their widespread acceptability. This may be the driving factor behind the current migration of common people to Ayurveda from the allopathic system.[6]

Since historical times, India is practicing the usage of medicinal herbs for the management and treatment of diabetes mellitus. Usage of herbs as hypoglycemic agents exerts a more beneficial effect as they consist of numerous phytoconstituents belonging to various classes such as alkaloids, flavonoids, steroids, glycosides, etc. Each phytocompound will exhibit its own mechanism of action. Hence, these phytocompounds may act synergistically to protect the β-cells during the diabetic condition and reduce the amount of glucose level in the blood.[7]

Over recent decades, the worldwide appeal of herbal medicines/products has expanded not only in the context of conventional treatment strategies but also in the area of healthcare management. However, the primary shortcomings of herbal medicines/products are;[8]

- Lack of quality assessment and Authentication of Herbal raw materials
- Adulteration
- Inefficient processing techniques leading to low yields.
- Poor quality control procedures and lack of appropriate standardization.
- Contamination with toxic substances mainly occurring from agricultural practices.
- Incomplete information about active principles present in finished herbal products.
- Scanty research work on herbal product and process development.

Hence, there is a need for thorough standardization and quality control of herbal drugs/ medicines.

The manufacturing process for herbal medicine products is complex, involving numerous unit operations. Starting from the raw material processing till the preparation of the final product, each step should be critically assessed and monitored in order to nullify the above lacunae. Scientific and systematic approaches for the development and optimization of herbal formulations should be employed for understanding the relationship between material attributes, process parameters, and product quality attributes in an efficient and satisfactory manner. According to the recent literature the Quality by Design (QbD) concept can serve as a novel approach to overcome the problems related to herbal drug development.[9, 10]

Nowadays, regulatory authorities are encouraging pharmaceutical companies to adopt novel approaches that can assure higher quality and product safety standards. The Quality by Design approach being the key enabler for achieving the desired product performance and quality in a quantum leap.[11] The QbD approach relies on planned, scientifically and systematically guided procedures, which enables the establishment of correlations between independent variables with dependent variables, which in turn leads to a sufficient understanding of the dependencies between the quality of the product and the process-formulation parameters in order to yield a quality product.[12]

ICH Q8 defines QbD as "A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management."[13]

Taking into account the fact that experience-based methods are dominantly used during the manufacture of herbal drug products, which fail to improve process understanding due to the complex chemical constitution and multiple process variables, it is therefore, significant to develop manufacturing process under the framework of QbD, which aims to move the formulation process away from empirical trial-and-error approaches and to orient the processes into predictable and precisely controlled environments to ensure product quality within the life cycle.[9,14] Consequently, QbD model must be implemented for the development of herbal medicines in order to achieve a significant level of safety, efficacy, and quality for commercial herbal products.[15]

1.2 JUSTIFICATION FOR STUDY

Regardless of great advances in the modern system of medicine, traditional medicine is still considered the primary form of treating diseases in the majority of countries. However, the lack of standard quality control profiles is the major focus of attention for herbal medicines. In such a situation, quality assessment of the herbal medicines/ products is a crucial prerequisite in order to ensure reproducible quality of herbal medicine which in turn will contribute to its safety and efficacy.

The development of Quality standards for herbal drugs is a challenging task and it needs innovative and creative approaches, different from the existing methods. Starting from the raw material selection, standardization, extraction to the formulation of the extracts into suitable dosage form, the problems vary with each plant species due to their complex nature. Quality control profiles must be established at each phase, and suitable strategies should be implemented to reduce batch-to-batch variation to maintain the quality of the product.

The need of the hour is to evolve a systematic approach for the standardization of herbal raw materials and herbal formulations. Concerning this, an attempt is being made to implement the QbD approach for the development of herbal medicines in order to achieve a significant level of safety and quality of the herbal products.

The QbD approach aims to move the formulation process away from empirical trial-and-error approaches and precisely control the environment to ensure product quality throughout the life cycle. By means of the QbD approach, quality is built into the process/product from the onset, thus counteracting the traditional quality by testing (QbT) approach, which tests product quality at the end of the manufacturing process.

The purity and quality of herbals are critical determinants of safety. Therefore an attempt is being made to assure the quality of herbal medicinal product starting from the raw material till the manufacture of the final herbal product by applying the QbD approach. Scientifically developed and standardized quality protocol for the development of the quality herbal product so produced with regard to the above project will play an important role in the future advancement of herbal medicines in healthcare.

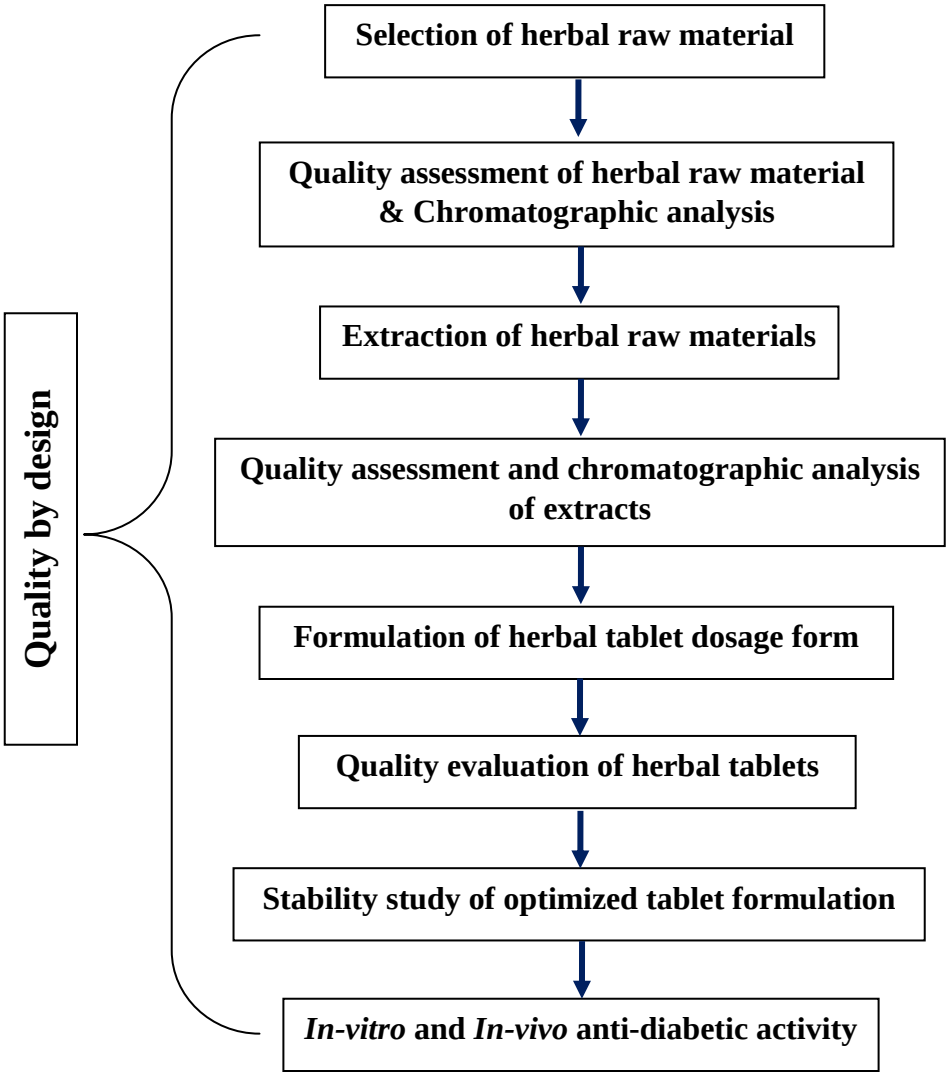
1.3 AIM, OBJECTIVES AND PLAN OF WORK

Aim: To formulate and evaluate an Anti-diabetic Herbal Drug Product by application of Quality by Design approach

Objective:

- To establish a Quality protocol for development of herbal drug product by application of Quality by Design Approach.
- Formulation and evaluation of an anti-diabetic herbal drug product with respect to above developed Quality Protocol.

PLAN OF WORK


Quality by design
Selection of herbal raw material
Quality assessment of herbal raw material & Chromatographic analysis
Extraction of herbal raw materials
Quality assessment and chromatographic analysis of extracts
Formulation of herbal tablet dosage form
Quality evaluation of herbal tablets
Stability study of optimized tablet formulation
In-vitro and *In-vivo* anti-diabetic activity

2. REVIEW OF LITERATURE

Herbs as a source of Anti-diabetic agents

Diabetes mellitus is one of the serious, chronic metabolic disorders affecting human health in both developing and developed countries. It is known to be the complex metabolic disease with numerous etiologies primarily marked with elevated blood sugar levels along with carbohydrate, protein, and lipid metabolism imbalances. Natural substances, specifically plants/ herbs are considered to play a crucial role in drug development research. Being the rich source of numerous phytoconstituents, plats serves as the primary source for discovering lead molecules with pharmacological benefits.[6] Plants have been regarded as a primary source of effective hypoglycemic medications for decades. Nowadays, herbal medicines are utilized for the treatment of diabetes by many developing nations, particularly to alleviate the cost, complications and side effects associated with conventional allopathic drugs.[7]

Gymnema sylvestre

Figure 1: *Gymnema sylvestre* leaves (wikipedia[16])

Family: Asclepiadaceae

Synonyms: Meshashringi, gurmar

Parts used: leaves

Phytoconstituents: Gymnemic acids, gymnemasaponins, gymnemosides, Gurmarin, etc.[17]

Uses: Anti-diabetic, diuretic, stimulates the heart and circulatory system.[17,18]

Anti-diabetic effects of *G. sylvestre*

Various *in-vitro*, *in-vivo*, and clinical investigational studies have suggested the anti-diabetic effects of *G. sylvestre* leaves. In an experimental study, Prabhu S. et al., investigated the effectiveness of *G. sylvestre* leaf extract on streptozotocin induced diabetic rats. Beneficial effect of the extract on blood glucose level and enhancement of serum insulin levels were reported.[19] Shenoy RS. et al., reported the usefulness of triterpenes glycoside isolated from *G. sylvestre* for the inhibition of pancreatic α -amylase, α -glucosidase, sucrase, and maltase.[20] The efficacy of the leaves extract in regenerating pancreatic β cells has been demonstrated in a study.[21] In a clinical study conducted by Gaytan Martinez L et al., reported increased insulin sensitivity in patients with impaired glucose tolerance by administration with *G. sylvestre*. [22]

Trigonella foenum graecum

Figure 2: *Trigonella foenum graecum* seeds (Parab Gaonkar et al.[23])

Family: Fabaceae

Synonyms: Methika, Methi, fenugreek

Parts used: seeds

Phytoconstituents: Trigonelline, Fenugreekine, Diosgenin, 4-hydroxyisoleucine, etc.

Uses: Appetite builder, Digestive, hypoglycemic, Mild laxative.

Anti-diabetic effects of *T. foenum graecum*

T. foenum graecum is widely used to manage diabetic conditions and its anti-hyperglycemic effects have been demonstrated by various experimental studies. In a study seed extract of *T. foenum graecum* was tested against streptozotocin-induced diabetic guinea pigs. The results showed the significant decrease in blood glucose levels and increase in serum insulin levels when compared to diabetic control animals indicating protective effects in diabetic conditions.[24] Trigonelline was found to have an inhibitory effect on the activity of glycogen synthase kinase isoforms in the regulation of glycogen metabolism, resulting in hypoglycemia.[25] Trigonelline has also shown enhancement of glucose and lipid hemostasis by improving the insulin signaling pathway.[26] In an multicentric Randomized control trial conducted by Verma N et al., reported the beneficial effects of novel *T. foenum graecum* extract in type 2 diabetes patients. The study resulted in decreased blood sugar and HbA1c levels and increased fasting and post-prandial C-peptide levels.[27]

Curcuma longa

Figure 3: *Curcuma longa* rhizomes (Gezici S.[28])

Family: Zingiberaceae

Synonyms: Haridraa, Haldi, turmeric

Parts used: rhizomes

Phytoconstituents: Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin, etc.

Uses: anti-inflammatory, antibacterial, antiviral, anti-diabetic, etc.

Anti-diabetic effects of *C. longa*

Rai P. et al., studied the effect of *C. longa* rhizome powder on STZ-induced diabetic rats and reported its positive effects in decreasing the blood glucose levels.[29] A study reported the significant improvement of glucose tolerance and insulin sensitivity by administration of curcumin enriched yougurt in diabetic rats indicating the anti-diabetic effects of curcumin.[30] Kuroda et al., reported that the hypoglycemic effect of *C. longa* is mainly attributed to PPAR-γ ligand-binding activity of the phytocompounds present in it.[31] Curcumin is also reported to inhibit glycogen synthase kinase 3β and produce hypoglycemic effect.[32] In a clinical study, Chuengsamarn S. et al., incorporated patients with curcuminoids capsule for 9 months

and observed the decrease in fasting plasma glucose and HbA1c and increased HOMA-β and better β cell Functions.[33]

Piper nigrum

Figure 4: *Piper nigrum* fruits

Family: *Piperaceae*

Synonyms: maricha, kali miri, Black Pepper.

Parts used: fruits

Phytoconstituents: Piperine, piperatine, piperidine, piperyline, piperoleins A and B, etc.

Uses: Stimulant, carminative, diuretic, Used in dyspepsia, indigestion, gastro-intestinal stimulant,flatulence, bioenhancer etc.

Anti-diabetic and Bio-enhancing effect of *P. nigrum*

In a study Atal S. et al., evaluated the blood glucose lowering effect of Piperine in alloxan induced diabetic rats. The results indicated significant blood glucose lowering effect of piperine in subacute study at dose of 20 mg/kg.[34] In a study conducted by Ciddi V. et al., combined piperine with glimpiride and studied the Pharmacokinetics and Pharmacodynamics of the drug in normal and diabetic rats. The results suggested the synergistic effect of piperine with glimpiride in decreasing the

blood glucose level and increasing the serum insulin levels in diabetic rats. Further indicating the bioenhancing effect of Piperine.[35] similarly, another study combining Piperine with metformin was conducted to evaluate the blood glucose lowering effect in diabetes mice. The study reported the significant reduction of blood glucose levels in diabetic mice treated with combination of Piperine and metformin when compared to mice treated with metformin alone suggesting the bioenhancing effect of Piperine.[36]

Quality assessment of herbal drugs

Quality assessment of herbal drugs is of paramount importance. The quality of herbal drugs can be characterized by defining the identity, purity, constituents present in them, and other physical, chemical, or biological attributes exhibited by them. The quality criteria for herbal medicinal products are dependent on a detailed scientific characterization of the raw material. The quality of herbal raw materials has a direct impact on the efficacy and safety of the final herbal medicinal products. Hence, Authenticity, purity, and assay are regarded as important aspects of the quality assessment of herbal drugs.[37]

With the aim of maintaining phytochemical consistency, safety and clinical efficacy of herbal medicines Govindaraghavan S. et al., outlined the steps involved in quality assessment of medicinal herbs.[38] A study has been conducted to develop quality control parameters for an ayurvedic formulation. The quality, safety and purity of the ayurvedic formulation were assessed by performing standardization and a monograph on quality standards was developed. TLC fingerprints were reported for authentication of the formulation.[39] Similarly, quality control parameters were

established for the standardization of *Ficus racemosa* L. and *Limonia acidissima* L. individually. Pharmacognostic parameters including macroscopy, microscopy, physicochemical, phytochemical and toxic contaminant analysis were reported.[40,41]

The most challenging area of quality control to accomplish is determining the content or assay of the herbal drugs. Since herbal drugs consist of various phytoconstituents identification and quantification of each one of them is a difficult task. Suitable analytical techniques and concepts need to be utilized for defining uniformity in herbal drugs. Hence, the Standardization of herbal drugs is considered as an important tool for ensuring the quality and optimum level of active principles for their bio potency.[37] Recently marker-based standardization of herbal drugs is gaining importance due to its ability to give an account of the phytoconstituents present in a particular plant and also to monitor batch to batch uniformity of phytoconstituents in the finished herbal products. Several chromatographic techniques ranging from simplest Thin Layer Chromatography to sophisticated High-Performance Liquid Chromatography (HPLC), High-Performance Thin Layer Chromatography (HPTLC), and Gas Chromatography (GC) can be utilized for such marker-based standardization of herbal drugs.[42,43] A study reported marker based standardization of Sarasvata Ghrita (SG) by utilizing HPTLC method for the estimation of Berberine, Piperine, 6-Shogaol and β- Asarone in marketed and in-house prepared formulations of SG.[44] Another study adopted HPLC method for performing marker based standardization of *Achyranthes aspera* by estimating betain in extracts and marketed formulations containing *Aaspera*.[43]

Along with the utilization of marker-based techniques, application of novel quality approaches are essential for quality assessment and development of standardization parameters for herbal drugs. In recent times, pharmaceutical companies adopting QbD as a fundamental pharmaceutical quality model for product development.[45] Analytical Quality by Design (AQbD) concept has been consistently utilized for the analysis of pharmaceutical products. Implementation of AQbD concept for standardization of herbal drugs can pave away for development of quality control parameters for herbal drug standardization. Moreover, AQbD approach for marker based analysis of herbs will help in developing reliable and robust methods with lesser time and cost for drug analysis.[46,47] Many AQbD case studies have been proposed indicating its efficiency in developing accurate and robust analytical methods for estimation of compound of interest.[48-50]

Extraction of Herbal drugs

Several extraction techniques are available such as cold maceration, soxhlet extraction, percolation, microwave-assisted-extraction, supercritical fluid extraction, etc. for obtaining the extract of herbs. However, soxhlet extraction is widely accepted method. In a study researchers have developed a novel extract of *G. sylvestre* leaves and claimed its ability to stimulate insulin secretion from human islets. The extraction procedure involved 40% ethanol as solvent for extraction of fresh leaves of *G. sylvestre.*[51] 70% ethanolic extract of *T. foenum graecum* is reported to improve glycemic functions in n-Stz diabetic rats.[52] Paulucci VP. et al., optimized the extraction method for curcumin from *Curcuma longa* L. Ethanolic strength as the extraction solvent was reported to be the most influential variable for the extraction.

Ethanolic strength of 70% and extraction temperature of 80ºC was reported to be the optimized extraction parameters.[53] Also, many studies have concluded 70% ethanolic extract of *C. longa* to show anti-hyperglycemic activity.[54,55] For the extraction of piperine from *P. nigrum* 90-95% of ethanol is considered as ideal solvent concentration for extraction.[56,57]

Formulation Development

Herbal medicinal products are available in various forms such as liquid, powder, capsule, tablet etc. Amongst these tablet formulations are considered as one of the most common and easy-to-use oral solid dosage forms.[58] Herbal medicines prepared in the form of solid dosage forms are usually prepared by using dried herbal extracts. These extracts are amorphous in nature and tend to absorb moisture. This property can be critical in terms of manufacturing process as well as for quality of finished product. Hence, selection of proper excipients and optimization is necessary to overcome the above issue during the manufacturing of tablet containing herbal extracts.[59]

A study developed herbal tablets from extracts of *Anogeissusleio carpus* and *Prosopis Africana.* Four different types of disintegrating agents were tested for their effects on tablet disintegration time and dissolution rate. It was reported that tablets incorporated with Explotab showed the lesser disintegration time whereas tablets incorporated with Prosolv showed the highest disintegration time.[60]

A study demonstrated the effect of formulation variables on tablet quality by performing statistical analysis. For the development of herbal tablets containing *Morus alba* leaf extracts statistical experimental design based Box-Behnken design, was

constructed. It was found that experimental statistical method was useful for selecting proper excipients and developing herbal tablet.[58]

Tablet formulations are conventionally prepared by wet granulation (WG), dry granulation (DG) and direct compression (DC) methods. Amongst this direct compression method is considered as the most economical technique to produce large batches of tablets. However its efficacy directly depends on the raw material attributes. Hence researchers have used Aeroperl® 300 Pharma as excipients for the development of *Silybum marianum* tablets. For the direct compression of tablets Aeroperl®300 Pharma is reported to be an an excellent pharmaceutical absorbent. Further, Co-processed excipients MicroceLac® 100 and FlowLac® 90 were utilized to improve compressibility and flowability.[61]

For the rapid disintegration of the herbal tablet crosscarmellose sodium was reported to be the ideal disintegrant. This was evident from the study conducted by Puri D. and co-workers in which they have developed herbal fast disintegrating tables. They compared and studied the effect of three different superdisintegrants which are sodium starch glycolate, crospovidone and croscarmellose and reported croscarmellose to be the optimized superdisintegrant.[62]

Quality by Design approach for Formulation development

Nowadays, regulatory authorities are encouraging pharmaceutical companies to adopt novel approaches that can assure higher quality and product safety standards. The QbD approach being the key enabler for achieving the desired product performance and quality in a quantum leap. The QbD concept is a systematic approach

which enables the development of product with predefined quality attributes and minimizes the defects in manufacturing process.[11]

Joseph M. Juran developed the QbD concept in 1992 to address quality control issues in manufacturing processes. As described in ICH Q8 guidelines QbD is "A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management."[13]

As per the traditional quality by testing (QbT) approach, only the final product is tested for the quality characteristics. More specifically, QbT is the concept that evaluates the quality of the product without looking at problems that may affect the quality of the final product during the initial production process. The QbD approach, on the other hand, recommends monitoring and assessing quality characteristics at every stage, beginning with product designing, processing, and manufacturing.[63]

In QbD concept, characteristics that are thought to be critical for the product quality are figured out, then transformed into attributes that the finished product should possess, and ascertain the variability among the critical process parameters to produce the drug product with predefined characteristics consistently.[64]

A successful implementation of QbD approach for development of orally disintegrating tablets (ODT) has been demonstrated in a research work. Ishikawa (fishbone) diagram and preliminary hazard analysis tool was adopted for performing risk assessment. Box–Behnken design was utilized for analyzing the effect of critical factors on various attributes of ODTs.[65]

Benjasirimongkol P. et al., utilized Quality by Design approach for development of Resveratrol Spray-Dried Emulsion. Process factors that affected the quality of the spray-dried emulsions were analyzed using Ishikawa diagram and risk ranking system. The effect of excipients used in the final product quality was evaluated by using the Plackett–Burman design. Further it was reported that the risk assessment and Plackett–Burman design mitigated the risks and identified the critical factors that affected the quality of the spray-dried emulsions of resveratrol and the spray-drying process.[66]

Another study demonstrates implementation of QbD concept for Identification, optimization and validation of critical process parameters like media milling and spray drying and critical formulation parameters (drug and excipients concentrations) for developing a nanocrystalline zileuton formulation. It was reported that QbD approach enabled the development of stable spray-dried nanocrystalline zileuton with exceptionally high total product yield and small particle size with low PDI.[67]

Parab Gaonkar et al., employed QbD approach for the development of herbal antioxidant formulation. QTPP and CQA for herbal supplements were defined. Risk priority numbers were assigned for the formulation parameters to analyzed the risk factors followed by execution of DoE by adopting central composite design. The effect of formulation variables such as concentration of starch and crosscarmellose sodium on disintegration time and friability was analyzed. The study suggested QbD approach as an effective approach for understanding the quality parameters for optimizing Herbal Supplement.[68] Similar study reported the optimization of herbal

tablets of *Psidium guajava*, *Mangifera indica*, and *Moringa oleifera* by application of QbD approach.[69]

In-vitro and *In-vivo* Anti-diabetic activity

Recently, a lot of research work is being carried out for exploring newer lead molecules of anti-diabetic potential. Subsequently, various *in-vitro* and *in-vivo* models have been developed for investigating the anti-diabetic efficacy of such compounds. *In-vitro* analysis is regarded as the initial step in the screening of potential anti-diabetic agents. Screening for enzyme inhibition, insulin sensitizing potential and insulin secretagogue activity are amongst the widely utilized tools currently for investigating anti-diabetic potential of plant based medicines. These are typically performed without the use of animals, using biochemical assays and cell lines, and are thus less expensive, more efficient, and easily adaptable for high-throughput screening.[70]

There are several enzymes that can be targeted for research in diabetes therapy. Inhibitions of carbohydrate digestive enzymes such as α-glucosidases and α-amylase are among the most effective therapeutic approaches for the management of hyperglycemia. Inhibiting these two enzymes will lead to significant reduction of blood glucose level. As a result, it serves as a useful strategy in the management of blood glucose levels in diabetic conditions.[71] Many investigations have been conducted to explore the α-glucosidases and α-amylase enzyme inhibitory effect of herbs.[72-75]

For *in-vivo* experimental studies animal models are one of the most important tools for moving forward with developing an effective model to investigate the

efficacy of herbs that have been claimed to have anti-diabetic properties. For most of the anti-diabetic investigations rodents are considered to be the ideal model however some of the experiments are still performed on larger animals. For evaluating the anti-diabetic activity of any compound, induction of diabetes mellitus in the animal is the prerequisite and this can be achieved by chemical, surgical and genetic manipulations.[76]

Majority of the research work on anti-diabetic activity has been conducted on chemically induced rodent models. Streptozotocin and Alloxan are widely used chemical compounds used for induction of Type II diabetes. It has been reported that most of the studies (69%) have been done on streptozotocin induced diabetic models when compared to alloxan induced diabetic model (31%). [77]

Streptozotocin (STZ) is a nitrosourea analogue that was discovered as an antibiotic and is also known for its cytotoxic properties.[78] It exerts a toxic effect on pancreatic β cells causing their destruction and thereby leading to suppression of insulin secretion.[76] It has been reported that Streptozotocin enters the pancreatic β cell via a glucose transporter-GLUT2 and causes alkylation of deoxyribonucleicacid (DNA). Furthermore, STZ induces activation of poly adenosine diphosphateribosylation and nitric oxide release. As a result of STZ action, pancreatic β cells are destroyed.[79] It is very easy and convenient method to induce experimental diabetes in rats by utilizing STZ as a single intra-paretoneal injection of compound can cause diabetic conditions in the animals. [78]

3. MATERIALS AND METHODS

Table 1: List of Herbal raw materials

Sr. No.	Raw material	Supplier
1.	*Gymnema sylvestre* leaves	B. M. K. Ayurveda Mahavidyalaya, Belagavi.
2.	*Trigonella foenum-graecum* seeds	B. M. K. Ayurveda Mahavidyalaya, Belagavi
3.	*Curcuma longa* rhizomes	B. M. K. Ayurveda Mahavidyalaya, Belagavi
4.	*Piper nigrum* fruits	B. M. K. Ayurveda Mahavidyalaya, Belagavi

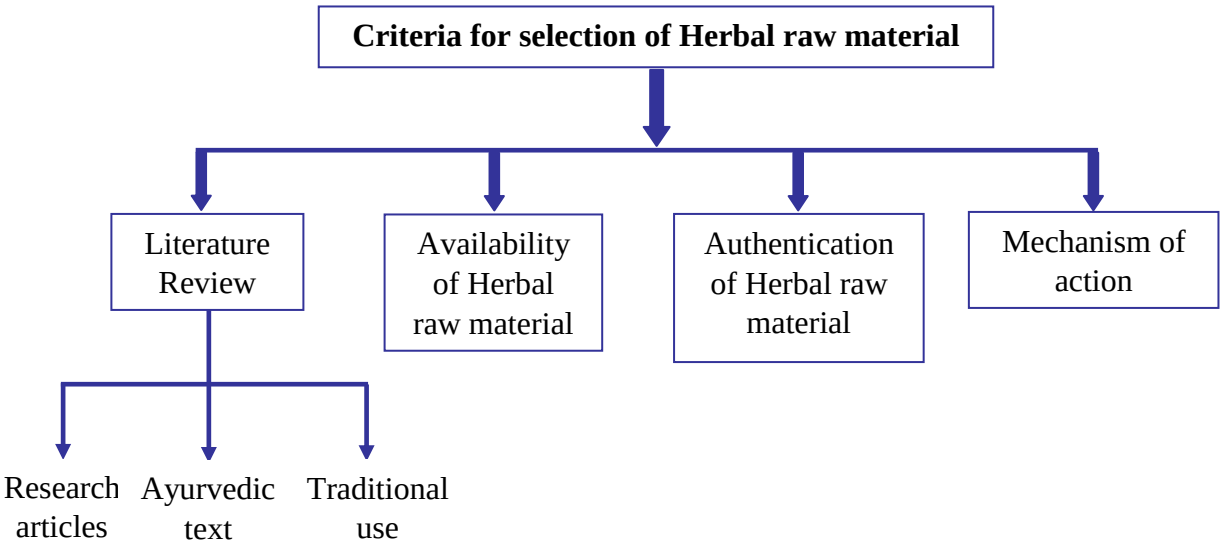
Table 2: List of Chemicals

Sr. No.	Chemical	Supplier
1.	Gymnemagenin	Natural Remedies Pvt. Ltd., Bengaluru
2.	Trigonelline hydrochloride	Himalaya drug company, Bengaluru India
3.	Curcumin	Himalaya drug company, Bengaluru India
4.	Demethoxycurcumin	Himalaya drug company, Bengaluru India
5.	Bis-demethoxycurcumin	Himalaya drug company, Bengaluru India
6.	Piperine	Himalaya drug company, Bengaluru India
7.	Orthophosphoric acid	Fisher Scientific, India
8.	Acetic acid	Fisher Scientific, India
9.	Acetonitrile	Fisher Scientific, India
10.	Methanol	Fisher Scientific, India
11.	Microcrystalline cellulose	Hi Media laboratories Pvt. Ltd.
12.	Crosscarmellose sodium	Hi Media laboratories Pvt. Ltd.
13.	Lactose anhydrous	Hi Media laboratories Pvt. Ltd.
14.	Streptozocin	Merck, India

Table 3: List of Instruments

Sr. No.	Instrument name	Model	Make
1.	Electronic Balance	AA-2130	Accord
2.	pH Meter	EQ-610	Equip-Tronics
3.	Trinocular microscope	METZ-780	Metzer
4.	Bath sonicator	CPX 1800 H-E	Branson
5.	High-Performance Liquid Chromatography	1220 infinity II LC	Agilent
6.	Rotavapor	RV 10	IKA, India
7.	FT-IR	IRAffinity-1S	Shimadzu
8.	Dissolution apparatus	USP Type 2	Electrolab India
9.	Disintegration Test Apparatus	ED-2AL	Electrolab, India.
10.	Roche Friability Tester	EF-2	Electrolab, India.
11.	Monsanto hardness tester	MHT-20	Campbell Electronics
12.	Vernier caliper	SERIES-530	Mitutoyo, Japan

3.1. Selection of Raw Material



Based on the above criteria the raw materials are selected by doing extensive literature review. By following the above criteria 50 herbs were reported as to possess anti-diabetic activity, which were identified and further reviewed thoroughly. Amongst the 50 reviewed herbs, 3-4 herbs having potent anti-diabetic activity were selected for the development of herbal anti-diabetic drug product.

3.2. Quality Assessment of Herbal Raw Material

3.2.1. Botanical Evaluation[80]

3.2.1.1. Macroscopic characteristics: Macroscopic characters of selected plant material were studied by examining various organoleptic parameters such as color, odor, taste, shape, and size.

3.2.1.2. Microscopic characteristics: Powder microscopy was performed on the powdered crude herbs for the determination of microscopic characters. Microscopic slides were prepared by staining the powdered plant material with phloroglucinol followed by the addition of a few drops of concentrated

hydrochloric acid. The photographs of specimens were captured by using the Trinocular microscope (Metzer) with Capture Pro software (4.6).

3.2.2. Physico-chemical Evaluation[80,81]

3.2.2.1. Moisture content: The moisture content of selected plant materials were determined by using Loss on drying (gravimetric determination) method.

3gms of the plant material was accurately weighed in a tared china dish and dried in an oven at 100-105°C until two consecutive weighings do not differ by more than 5mg and calculated the percentage loss of weight.

3.2.2.2. Extractive value: Solvent extractive values were determined by cold maceration method. 4gm of coarsely powdered plant material was macerated with 100ml of the specified solvents (water, ethanol and ether) in a glass-stoppered conical flask for 24 hrs with intermittent shaking for first 6hrs, and then allowed to stand for 18 hours. It was then filtered and 25 ml of the filtrate was transferred to a flat-bottomed dish and evaporated to dryness on a water bath and weighed. The percentages of extractable matter in the specified solvents were calculated with reference to the air dried drug.

3.2.2.3. Total Ash value: accurately weighed 2gm of the powdered plant material was placed in a previously ignited and tarred crucible. The crucible was then placed in a muffle furnace and ignited at 500-600°C until it is white, indicating the absence of carbon. It was cooled in a desiccator, weighed and subsequently the content of total ash was calculated in terms of percentage.

3.2.2.4. Acid-insoluble ash: 25 ml of hydrochloric acid was added to the crucible containing the total ash, and covered with a watch-glass and boiled gently for 5

minutes. The solution was filtered and the insoluble matter was collected on an ashless filter-paper and transferred to the original crucible. The crucible was then, ignited to constant weight, cooled in a desiccator and reweighed. The acid-insoluble ash content was calculated and reported in percentage.

3.2.2.5. Water-soluble ash: the total ash from the plant material was dissolved in 25 ml of water and boiled for 5 minutes. The solution was filtered and the insoluble matter collected on the ashless filter paper was further ignited in a crucible for 15 minutes at a temperature not exceeding 450°C. The residue was cooled in desiccator and reweighed. Weight of this residue was subtracted from the weight of total ash and the content water soluble ash was calculated in percentage.

3.2.3. Chemical evaluation

3.2.3.1. Phytochemical analysis: The powdered crude drugs were subjected to preliminary phytochemical analysis. In order to assess the qualitative chemical composition of the selected plant material by standard methods.[82] The crude drug was analyzed for the presence of secondary metabolites such as Alkaloids, flavonoids, tannins, phenols, saponins, glycosides, and steroids.

3.2.3.2. Aflatoxins determination: The presences of Aflatoxins B1, B2, G1 and G2 in the plant materials were determined as per standard procedure by the HPLC method.

Accurately weighed 5gm of each powdered raw material was transferred to a glass stoppered conical flask and 100mL of 70% methanol was added. The mixture was shaken for 30 min and then centrifuged at 1000 rpm. The

supernatant was collected and filtered through a 0.22 μm filter. 5ml of filtered aliquot was diluted with PBS buffer and passed through Immuno-affinity column (IAC) keeping the flow rate at 1 mL/min. The column was washed with 20 mL distilled water and then eluted with methanol and water in the ratio of 2:1 and the elute was collected. The elute was filtered through 0.22 μm filter and 20 μL of the sample was injected into HPLC system with C18 ODS2 column. The mobile phase employed was water: acetonitrile: methanol (55:15:30v/v). The Aflatoxins in samples were identified by comparing the retention times with the standards.

3.2.3.3. Determination of Pesticide Residue: The raw plant samples were analyzed for the presence of 17 different pesticide contaminants by Gas Chromatography-Mass Spectroscopy (GC-MS).

5 gm of each powered air-dried plant sample was taken in a centrifuge tube. 10 mL deionized water was added to it and shaken for few seconds and left to hydrate for 15minutes. Then 10 ml of acetonitrile was added to the mixture and Shaken by vortex and allowed to stand for two hours with constant shaking. The subsequent mixture was then added to the QuEChERS kit for extraction. The extracted mixture was vortexed and centrifuged for 5mins. Further, 1ml of the extract was transferred to clean up column and elute was collected, filtered and evaporated to dryness. The residue was further dissolved in hexane and injected to GC-MS instrument.

3.2.3.4. Determination of Heavy metals: Analysis of heavy metals was carried out by Atomic Absorption Spectroscopy (Perkin Elmer-400, carrier gas-Argon, flow

rate- 2 mL/3 min) by following standard method.[83] Presence of Heavy metals namely lead, cadmium, arsenic, mercury and chromium were tested in the crude powdered sample.

The preparations of samples were carried out by dry ashing method. 1gm of each herbal sample was weighted into a porcelain crucible and dry ashed in a muffle furnace by stepwise increase of temperature up to 500ºC within 1 h. The sample was ashed for approximately 4 h until a white or grey ash residue was obtained. The residue was dissolved in 1 M nitric acid, filtered into a 25mL volumetric flask using whatman filter paper and made up to mark with the nitric acid (1 M). The blank digests was similarly processed.

3.2.4. Biological Evaluation

3.2.4.1. Evaluation of Microbial load:

The microbial load present in the herbal samples was evaluated to assess the quality of the selected herbs. For microbial load, 1gm of each powdered plant material was suspended in sterile water, thoroughly mixed and the mixture was filtered. 1ml of the threefold diluted samples was inoculated aseptically on petri plate containing nutrient agar medium by spreading technique and incubated at 37ºC for 24 hours. The number of colonies on the petri plates were determined and microbial load was expressed in terms of colony forming units (CFU).

3.2.5. Chromatographic analysis

High Performance Liquid Chromatography (HPLC) method was utilized for the quantification of phytoconstituents in each selected herbal raw material. Analytical

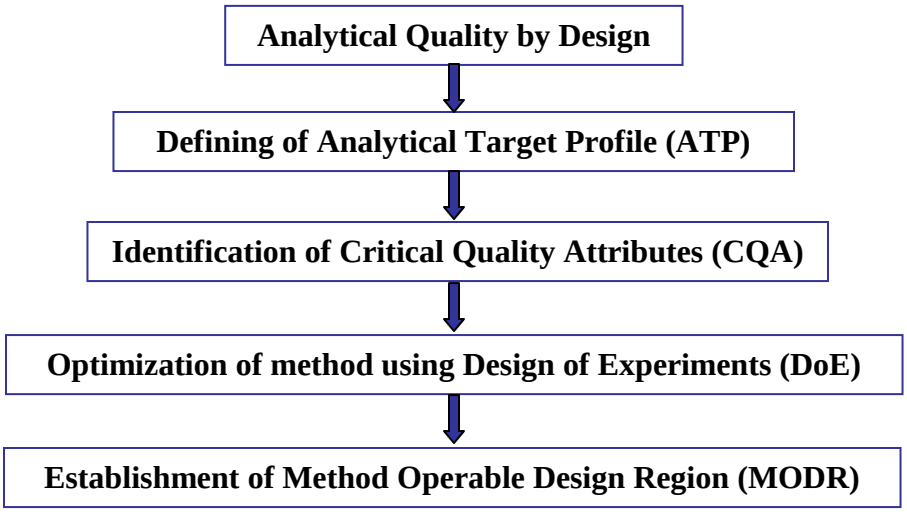
Quality by Design (AQbD) approach was employed for the method development and validation of each phytoconstituent in the raw material.

Based on the literature review the phytoconstituents specific to the selected plant and reported to be responsible for hypoglycemic effect of the herb was selected for chromatographic analysis. Hence, the phytoconstituents selected for are as follows,

Table 4: Phytoconstituents selected for HPLC analysis

Herbal raw material	Phytoconstituent/ marker compound
G. sylvestre	Gymnemagenin
T. foenum graecum	Trigonelline
C. longa	Curcumin
	Demethoxycurcumin
	Bis-demethoxycurcumin
P. nigrum	Piperine

AQbD flow-work for HPLC method development



3.2.5.1. Instrumentation and chromatographic conditions

HPLC system (Agilent technologies 1220 Infinity II LC) used for the analysis consisted of a system controller, low-pressure gradient pump, solvent delivery module, online degasser, manual sample injector (injection volume ranging between 5 and 20µL), and UV-Vis detector. Reversed-phase C-18 column (5µm, 4.6mm, 250mm, ZORBAX) was used for chromatographic separation.

3.2.5.2. Preparation of Standard and sample solution

A stock solution consisting of 1000µg/mL of each marker compound i.e., Gymnemagenin (GYM), Trigonelline (TRG), Curcumin (CUR), Demethoxycurcumin (DMC), Bis-demethoxycurcumin (BDC), and Piperine (PRN) was prepared using methanol. Further, different dilutions with varying concentrations were prepared using the respective mobile phase and filtered through a 0.22µm membrane filter prior to their injection into the chromatographic column.

In case of *G. sylvestre*, the acid-base hydrolysis method reported by Ahamad et al.,[84] was followed for sample preparation. Accurately weighed 500 mg of crude powdered *G. sylvestre* leaves were dissolved in 50ml of 50% ethanol. To this solution 10 mL of 12.0% KOH was added and heated on boiling water bath for 1 hr. After cooling, 9mL 4N HCl was added and heated on a boiling water bath for 1 hr. After cooling, 12.0% KOH was added to make pH between 7.5 and 8.5. Further the solution was filtered and diluted to 100ml. This sample solution was further used for HPLC analysis.

For the preparation of the sample, accurately weighed 1gm of powdered samples of *T. foenum graecum* seeds, *C. longa* rhizomes and *P. nigrum* fruits were refluxed for 1

hr with 50mL of methanol. The solution was filtered and diluted to 100 mL with methanol and were used for further analysis.

3.2.5.3. Defining of Analytical Target Profile (ATP) and Critical Quality Attributes (CQA)

Development of analytical method by application of the QbD approach necessitates the prior outlining of Analytical Target Profiles (ATP). ATP serves as the objective or goal of the method development process which needs to be achieved in order to accomplish reliable results. Critical Quality Attributes (CQA) are the quality characteristics related to method performance. CQAs have to be identified from the defined ATP which will be useful in ascertaining the satisfactory performance of the developed method. [48,85]

3.2.5.4. Optimization of the method using design of experiments (DoE)

A 2^2 full factorial design consisting of 2 factors and 4 experimental runs was chosen for identifying and associating the effect of independent variables on the CQA i.e. the selected dependent variables. The design of the experiments (DoE) was executed with Design-Expert software version 12.0, (Stat-Ease Inc., Minneapolis, MN, USA). The values arising out of the experimental trials were statistically evaluated by performing analysis of variance (ANOVA) and deriving polynomial equations. The relationship between the independent and dependant variables were finally empathized with the help of 3-D response surface plots.

3.2.5.5. Establishment of a method operable design region (MODR)

The optimized chromatographic conditions were predicted from the Method Operable Design Region (MODR). Taking into consideration the criteria's of ATP, method parameters such as lesser tailing factor, retention time, and peak width. An ideal mobile phase combination was identified from the overlay plot showing MODR. The yellow shaded region within the overlay plot represents the MODR in which all the specifications mentioned in the ATP are fulfilled at a specified risk level.

3.2.5.6. Quantitative estimation

The optimized RP-HPLC method was further applied for the quantification of each phytocompound in their respective plant material.

3.2.5.7. Analytical Method Validation

The optimized RP-HPLC methods were validated as per ICH Q2 (R1) guidelines.[86] The described methods were validated in terms of following parameters.

a. System suitability

System suitability was determined to evaluate the capability of the system to carry out the analysis. The % RSD of the parameters such as retention time, peak area, and tailing factor were determined by injecting the specific concentration of the analyte in hexaplicate.

b. Linearity

For developing the linearity range, different concentrations of each standard i.e. GYM, TRG, CUR, DMC, BDC, and PRN were analyzed and a calibration curve was plotted for each concentration corresponding to the obtained peak area. The regression

analysis for the obtained data was studied to establish the calibration equation and correlation coefficient (r^2).

c. Limit of detection and limit of quantification

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined as; LOD = 3.3 σ/s and LOQ = 10 σ/s where, σ is the standard deviation of y-intercepts and s is the slope of the calibration line.

d. Precision

The intra-day and inter-day precision for the optimized method was evaluated by determining the repeatability of the results exhibited by the analyte in terms of % RSD. Intra-day precision was performed three times in a day whereas the inter-day precision on three different days. The analyte was tested in triplicates.

e. Accuracy

The accuracy of the optimized method was evaluated in terms of % recovery. A known concentration of the standard solution was spiked on the samples of raw materials in triplicate injections. The accuracy was determined at three concentration levels i.e. at low, medium and high - 80, 100, and 120%, respectively. The mean percentage recovery for each standard was calculated.

3.3. Extraction

The extraction of each crude drug was carried out by cold maceration followed by soxhlet extraction method. Ethanol and Water was selected as solvents for the extract preparation due to their non-toxic nature. Based on literature review the ratios of ethanol and water for each crude drug were selected in order to extract the phytocompounds of interest and to yield maximum amount of extract.

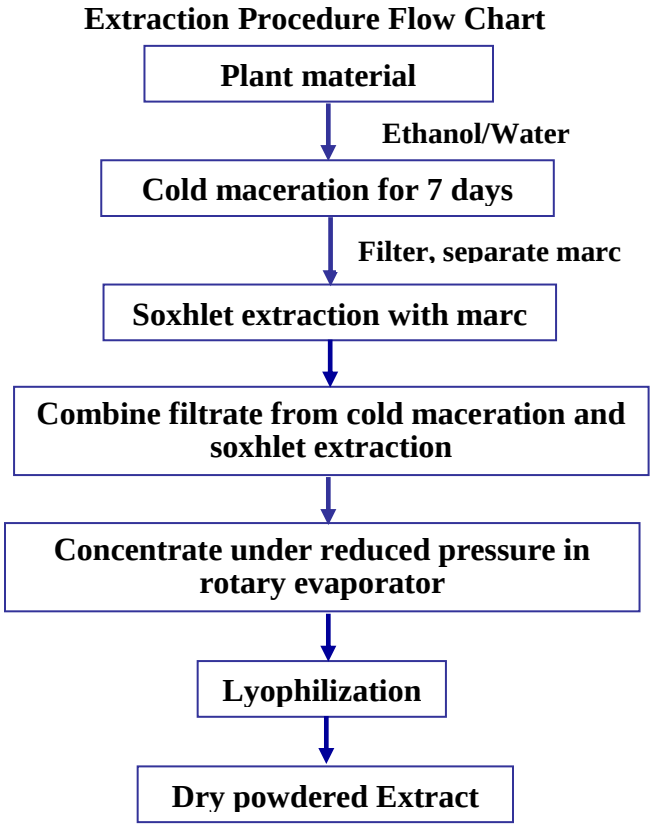


Table 5: Crude drug and solvent ratio for extraction

Crude drug	Extraction solvent
Dried leaves of *G. sylvestre*	Ethanol: Water (60:40)
Dried seeds of *T. foenum graecum*	Ethanol: Water (70:30)
Dried rhizomes of *C. longa*	Ethanol: Water (70:30)
Dried fruits of *P. nigrum*	Ethanol: Water (90:10)

3.4. Quality Assessment of Herbal Drug Substance (Extract)

3.4.1. Moisture content

The moisture content of selected plant materials were determined by using Loss on drying (gravimetric determination) method.[80]

3.4.2. Flow Properties

The flow property or powder fluidity is one of the important requirements of a drug substance which decides its utility and application in the development of different dosage forms. The flow properties of the powdered extracts were evaluated by determining its bulk density, tapped density and angle of repose, using methods reported in the literature.[87]

3.4.3. Phytochemical analysis

The powdered extracts were subjected to phytochemical analysis by standard methods.[82] The crude drug was analyzed for the presence of secondary metabolites such as Alkaloids, flavanoids, tannins, phenols, saponins, glycosides, and steroids.

3.4.4. Chromatographic analysis

Each plant extract was subjected to HPLC analysis to determine the quantity of active phytocompounds (marker) present in them after extraction. The developed HPLC methods were utilized for the quantitative estimation of Gymnemagenin, Trigonelline, Curcuminoids and Piperine in *G. sylvestre, T. foenum-graecum, C. longa* and *P. nigrum* respectively.

3.4.5. *In-silico* molecular docking

The *In-silico* molecular docking studies have been carried out to predict the binding affinity of bioactives from the selected plants against α-Glucosidase and α-amylase enzymes, which are the key enzymes in post-prandial hyperglycemia. The important bioactive compounds such as Gymnemagenin, Trigonelline, Curcuminoids,

and Piperine from *G. sylvestre*, *T. foenum graecum*, *C. longa* and *P. nigrum* respectively were docked with the two enzymes α-Glucosidase and α-amylase.

The three dimensional (3D) structures of the selected six bioactives were retrieved from PubChem chemical database in .sdf format and converted into .pdb using Discovery studio 2020. Then the energy of each compound was minimized using uff forcefield and converted into ligand molecules. Two enzymes, α-glucosidase (PDB:3TOP) and alpha-amylase (PDB: 4W93) were retrieved from RCSB protein databank. Further, the docking was performed by using autodock4.0 tools in PyRx0.8 platform. After docking 10 different poses of ligands were obtained. The pose of ligand with minimum binding energy was chosen to visualize the ligand-protein interaction in Discovery Studio 2020.

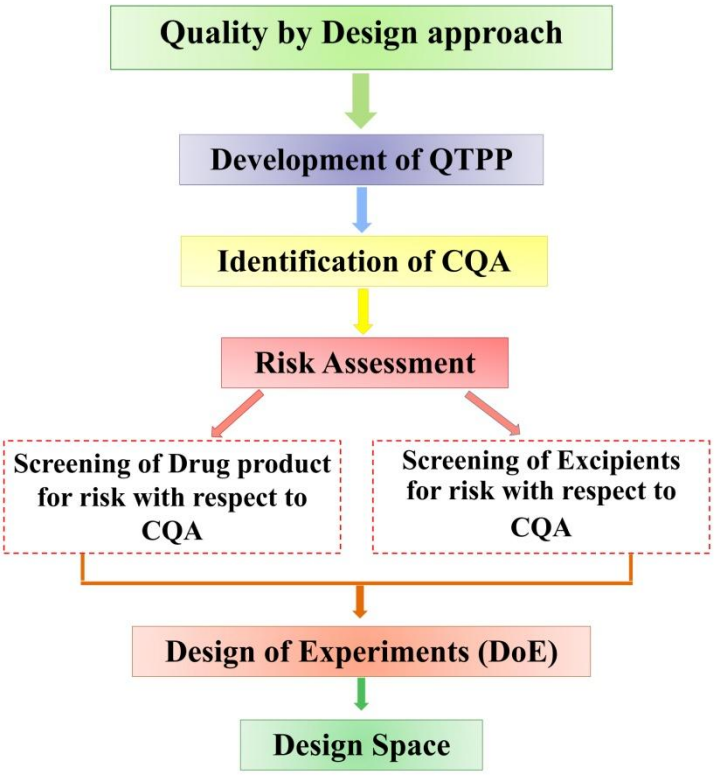
3.5. Formulation Development

3.5.1. Compatibility study

Compatibility of the herbal extracts with excipients was investigated by FT-IR spectroscopic analysis. IR spectral analyses were carried out by potassium bromide method.

3.5.2. Quality by Design approach for Formulation development

QbD flow work for formulation development



3.5.2.1. Development of Quality Target Product Profile (QTPP)

Development of Quality Target Product Profile (QTPP) is the first and most essential step of QbD approach. It begins with the definition of the desired product attributes that are needed to ensure equivalent quality and safety. QTPP deals with the quality characteristics that certify target product profile on product label. It acts as a guide to the product development, setting the target or goal in advance.

3.5.2.2. Identification of Critical Quality Attributes (CQA)

After the development of QTTP, the second step is identification of critical quality attributes (CQA). CQAs are the parameters that influence the QTPP. CQAs are

highly dependent on the critical material attributes (CMA) of active ingredient and excipients used in the formulation of product as well as critical process parameters (CPP) during manufacturing. The CQAs were defined from QTPP to identify satisfactory quality of the product.

3.5.2.3. Risk Assessment

Risk assessment (RA) is the most important element of QbD based formulation development. RA being a science-based approach is utilized in quality risk management that identifies the relationship between critical material attributes (CMA) and Critical process parameters (CPP) with that of product CQAs. The initial risk assessment for critical input material and formulation components was conducted by constructing an Ishikawa diagram and a relative risk-ranking system.

3.5.2.4. Design of Experiments

Design of Experiments (DoE) is the key element of QbD. The potential factors having a high impact on CQAs resulting from the risk assessment program were evaluated by using DoE to find the optimized product manufacturing environments.

A 3^2 full factorial design was used for the screening of potential high risk factors obtained from risk assessment. In the present design two high risk factors (Independent variables) were evaluated at three levels and experimental trials were performed. Two independent variables; concentration of binder i.e. Microscrystalline cellulose (MCC) - X1 and concentration of disintegrant i.e. Crosscarmellose sodium (CCS) -X2 were varied at 3 different levels that were coded for low (-1), medium (0) and high (+1) resulting in a total of 9 formulation batches. The dependent variables or response variables selected for

the present study were; Disintegration time -Y1, Hardness -Y2, and % Friability -Y3. The experiments were designed by using Design Expert software version 13.0, (Stat-Ease Inc., Minneapolis, MN, USA).

Table 6: Experimental design for formulation of Herbal Anti-diabetic tablets

Code	Coded levels		Actual values (mg)	
	Conc. of MCC X1	Conc. of CCS X2	Conc. of MCC X1	Conc. of CCS X2
F1	-1	-1	50	10
F2	+1	-1	100	10
F3	-1	+1	50	30
F4	+1	+1	100	30
F5	-1	0	50	20
F6	+1	0	100	20
F7	0	-1	75	10
F8	0	+1	75	30
F9	0	0	75	20

3.5.2.5. Formulation of Herbal Anti-diabetic tablets

Herbal anti-diabetic tablet formulation (650mg) was prepared by direct compression method. The dry powdered standardized extracts of *G. sylvestre* (150mg), *T. foenum-graecum* (150mg), *C. longa* (150mg) and *P. nigrum* (50mg) were used as active ingredients. Directly compressible microcrystalline cellulose was used as binder, crosscarmellose sodium was used as disintegrant, anhydrous lactose as filler. Total 500mg of the standardized extracts were thoroughly mixed with anhydrous lactose to control the moisture absorbing nature of the extracts. Further, to this mixture MCC and CCS were added and thoroughly mixed for 10 minutes. The quantity of the binder and

disintegrant were varied (low, medium, high) in the formulation batches as per DoE. Magnesium stearate and talc were uniformly mixed with the above mixture as lubricant and glidant respectively. The blend was compressed using Rimek single Rotary tableting machine equipped with 12mm biconvex faced punches to a target weight of 650 mg/Tablet.

Table 7: Formulation trial table

Ingredients (mg)	F1	F2	F3	F4	F5	F6	F7	F8	F9
G. sylvestre extract	150	150	150	150	150	150	150	150	150
T. foenum- graecum extract	150	150	150	150	150	150	150	150	150
C. longa extract	150	150	150	150	150	150	150	150	150
P. nigrum extract	50	50	50	50	50	50	50	50	50
Microcrystalline cellulose	50	100	50	100	50	100	75	75	75
Crosscarmellose sodium	10	10	30	30	20	20	10	30	20
Anhydrous lactose	85	35	65	15	75	25	60	40	50
Talc	03	03	03	03	03	03	03	03	03
Magnesium stearate	02	02	02	02	02	02	02	02	02

3.5.2.6. Pre-compression parameters (Micromeretics study)

Pre-compression parameters were performed for the powder blend of extracts and excipients so as to determine the flow properties of the formulation mixture. The pre-compression parameters studied were Bulk density, Tapped density, Angle of repose, Compresibility index and Hausener's ratio. All the parameters were performed by following standard procedure.[87]

i. **Bulk density:** was determined by pouring a weighed quantity of powder blend into graduated cylinder and measuring the volume and weight.

$$BD = \frac{weight\ of\ powder}{volume\ of\ packing}$$

ii. **Tapped density:** was determined measuring the weight of powder blend against tapped volume of the blend after tapping.

$$TD = \frac{weight\ of\ powder}{volume\ of\ tapped\ packing}$$

iii. **Angle of repose:** was determined by funnel method and was calculated by using following formula.

$$tan\theta = {}^{h}/_{r}$$

Where; h is height of powder cone formed and r is radius of the base of powder cone

iv. **Compressibility index:** The Compressibility index of the blends was determined by Carr's compressibility index.

$$Carr's\ Index = \frac{(TD - BD)}{TD} \times 100$$

v. **Hausner's ratio:** It is the measurement of frictional resistance of the drug. It is calculated using the formula below:

$$Hausner\ ratio = \frac{TD}{BD}$$

3.5.2.7. Quality Evaluation of Tablets

The tablets from all the batches were evaluated for Unofficial and Official Quality Control tests as per standard procedures.[88] The prepared tablets were evaluated for hardness, thickness, weight variation, friability, disintegration time, and, moisture content.

i. **Hardness:** hardness indicates the crushing strength of the tablets. The Monsanto Hardness Tester was used to check the hardness of tablets drawn at random from each formulation batch.

ii. **Thickness:** The thickness of the tablets was measured with a vernier caliper. Thickness of three tablets from each batch was measured and average values were calculated.

iii. **Weight Variation test:** Every individual tablets in a batch should be in uniform weight and weight variation must be within permissible limits. By randomly selecting and weighing 20 tablets, the average weight was determined. The tablets were also weighed individually and the percentage of deviation of its weight from the average was determined for each tablet.

iv. **Friability:** The friability test was performed by using calibrated Roche Friabilitor. Pre-weighed sample of 10 tablets was placed in the Roche Friability tester, which was then operated for 100 revolutions. Tablets were removed from Roche Friabilitor after 100 revolutions, dedusted and reweighed; tablets should not lose more than 1% of their initial weight.

$$\%\ Friability = \frac{Initial\ Wt - Final\ Wt}{Initial\ Wt} \times 100$$

v. **Disintegration Time:** *In-vitro* Disintegration time was performed by using Electrolab Disintegration tester. One tablet was placed in each of the six tubes of the basket. The apparatus was operated using deionized water as the immersion fluid maintained at 37±0.5°C. The time taken for complete disintegration of the tablet with no passable mass remaining on the mesh in the apparatus was measured in minutes.

vi. **Moisture content:** The moisture content of selected plant materials were determined by using Loss on drying (gravimetric determination) method.[83]

3.5.2.8. Statistical Optimization

The Statistical optimization was carried out to evaluate the goodness of fit of the model.[68] The values arising out of the experimental trials were statistically evaluated by performing ANOVA and deriving polynomial equations. The relationship between the independent and dependant variables were finally empathized with the help of contour and 3-D response surface plots.

3.5.2.9. Establishment of Design Space

Design space is the result of multidimensional combination and interaction of input variables, such as material attributes and process parameters, on quality of the product. It is the region meeting all the criteria's of CQA and with the predefined specifications of QTPP. Based on the specifications of CQA and QTPP, the optimized formulation batch was predicted from the developed design space.

3.5.2.10. *In-vitro* drug release study

Since, the formulated anti-diabetic tablets are the mixture of four herbal extracts the dissolution rate of the tablet was assessed by taking into consideration the specific marker compound from each extract. Hence, release rate of Gymnemagenin (*G. sylvestre*), Trigonelline (T. *foenum-graecum*), Curcumin (*C. longa*), Demethoxycurcumin (*C. longa*), Bis-Demethoxycurcumin (*C. longa*) and Piperine (*P. nigrum*) were studied.

In-vitro drug release profile of the optimized formulation batch was determined using USP Dissolution Apparatus II (paddle type) at a speed of 75 rpm, with 900 ml of the simulated gastric fluid (pH 1.2) and 0.5% sodium lauryl sulphate maintained at 37± 0.5ºC.[89] 5 ml of the samples were withdrawn at 15, 30, 60, 90, 120, 150 and 180 min time intervals with replacement of same volume of simulated fluid, respectively. The

samples were filtered through 0.45µ filter and were subjected to HPLC analysis for determining the amount of each marker compound released from the tablet formulations.

3.5.2.11. Drug Content

The assay of the optimized tablet formulation was carried out by HPLC analysis using the developed methods. The percentage content of each phytoconstituents (marker compound) in the tablet was determined.

Ten tablets from optimized formulation batch F3 was finely powdered and the amount of the powdered tablet equivalent to 10mg of each herbal extract was weighed accurately and added to 5ml methanol and sonicated for 15 minutes. The solution was diluted up to 10ml with methanol and was filtered through 0.45µ filter. From the above solution further dilution was made to a final concentration of 10µg/ml and injected into the HPLC system. From the peak area obtained the content of each marker compound per tablet was calculated and was expressed in terms of percentage (%) drug content.

3.5.2.12. Stability study

The optimized formulation was subjected to stability studies as per ICH Q1A (R2) guidelines.[90] The samples were placed in closed amber colored bottles and were stored at 25°C ± 2°C/60± 5% RH and in a humidity chamber maintained at 40 ± 2º C and 75 ± 5% RH for a period of three months. The tablet samples were analyzed for appearance, color, disintegration time, hardness and friability.

3.6. *In-vitro* anti-diabetic assay

The *in-vitro* anti-diabetic potential of the optimized herbal tablet formulation was evaluated by performing α-glucosidase and α-amylase inhibitory activity.

3.6.1. *In vitro* α-glucosidase inhibitory activity

In vitro α-glucosidase inhibitory activity was performed as explained by Khanal P. et al.[91] Briefly, different concentrations(50-500µg/ml) of optimized formulation F3 were tested for α-glucosidase inhibitory activity using p-NPG as a substrate and compared with standard acarbose. Experiment was performed in triplicates and percentage inhibition was calculated using formula (1).

3.6.2. *In vitro* α-amylase inhibitory activity

In vitro α-amylase inhibitory activity was performed as explained by Khanal P. et al.[92] Different concentrations of optimized formulation F3 were tested for α-amylase inhibitory activity; compared with standard acarbose using 1% soluble starch as a substrate. All the experiments were performed in triplicates and percentage inhibition was calculated using the following formula (1).

$$\%\ inhibition = \frac{Absorbance\ \ of control - absorbance\ \ of sample}{absorbance\ \ of control} X\ 100 \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

3.7. *In-vivo* anti-diabetic activity

In-vivo anti-diabetic activity of the optimized tablet formulation was performed on the streptozocin induced diabetic rat model to evaluate the effectiveness of the formulation in alleviating diabetic condition.

Animal study was performed after receiving ethical clearance from Institutional Animal Ethics Committee at KLE College of Pharmacy Belagavi (KLECOP/CPCSEA-Reg,No. 221/Po/Re/S/2000/CPCSEA, Res. 30–13/03/2021). Animals were procured from a CPCSEA registered vendor and were acclimatized for a week in the pathogen-free environment before performing the study. Thirty six, adult, albino rats of Wistar strain (210 ± 10 g) were used for the anti-diabetic study.

3.7.1. Induction of diabetes

Streptozocin at a dose of 35 mg/kg was injected through intra-peritoneal route for induction of diabetes. The streptozocin solution was freshly prepared by dissolving in chilled citrate buffer (pH 4.5). Rats with fasting blood glucose level of >200 mg/dL was included in the study.

3.7.2. Dose preparation

The optimized tablet formulation was suspended in vehicle i.e., RO water for the assessment of Anti-diabetic activity. Based on previous literature the LD50 of each herbal extract was greater than 2000 mg/kg. Hence, 1/5th, 1/10th and 1/20th of 2000 mg/kg were considered as the dosage for anti-diabetic activity. Therefore, 100, 200, and 400 mg/kg were selected as the per oral (p.o) dose for treatment groups.

3.7.3. Experimental design

A total of 36 rats (6 normal; 30 STZ-diabetic rats) were used in the study, which were dived into 6 groups containing 6 animals in each. Group 1: Normal control (NC) rats, administered with vehicle p.o., Group2: Diabetic control (STZ) rats administered with vehicle p.o., group 3: positive control (STZ+GLIB) treated with glibenclamide

5mg/kg, p.o., group 4: treatment group (STZ+F100) treated with formulation at lower dose of 100 mg/kg, p.o., group 5: treatment group (STZ+F200) treated with formulation at median dose of 200 mg/kg, p.o., and group 6: treatment group (STZ+F400) treated with formulation at higher dose of 400 mg/kg, p.o. All treatments were given orally after the 4th day of STZ administration for 28 days.

3.7.4. Study Parameters

The body weight, food intake and water intake was recorded 0, 7, 14, 21 and 28 day of the study. The % change in body weight, food intake and water intake was also calculated. The fasting blood glucose level was measured using a glucometer (Janaushadi, India). After the successful completion of the treatment, animals were fasted overnight for the estimation of fasting blood glucose level. Further, oral glucose tolerance test OGTT was performed to assess the glucose clearance by administering the 4 g/kg of exogenous glucose and the blood glucose level was recorded from 0 to 120 min at 30 min interval using a glucometer.

The animals were than sacrificed using mild anesthesia; the blood was collected via the cardiac puncture and serum was separated for the estimation of biochemical parameters such as high-density lipoprotein (HDL), triglycerides (TG), and total cholesterol (TC), Low density lipoprotein (LDL) and very Low density lipoprotein (VLDL) using commercially available kits. Also, pancreas was isolated for the histopathological examination.

3.7.5. Histopathology

For the histopathological examination, the pancreas was fixed with 10% formalin, sectioned, stained with hematoxylin and eosin (H & E) and observed for histopathological changes.

3.7.6. Statistical analysis

All the data are presented in mean±SEM (n=6). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test.

4. RESULTS

4.1. Selection of Raw Material

Based on the criteria defined for the selection of raw material 50 herbs reported to possess anti-diabetic activity were identified. Further, extensive literature search on the identified 50 herbs was carried out mainly focusing; a) quality and authenticity, b) efficacy, c) mechanism of action and synergistic effect, and d) availability of herbs, its marker compound, and cost. Finally three herbs having potent anti-diabetic activity and additional herb acting as a bio-enhancer was selected for the development of anti-diabetic formulation. Table 8 enlists the selected herbs.

Table 8: Herbal raw materials selected for development of anti-diabetic formulation

Herb	Family	Active principle	Biological activity
Gymnema sylvestre	Asclepidaceae	Gymnemic acids	Anti-diabetic
Trigonella foenum graecum	Fabaceae	Trigonelline	Anti-diabetic
Curcuma longa	Zingiberaceae	Curcuminoids	Anti-diabetic
Piper nigrum	Piperaceae	Piperine	Bio-enhancer

4.2. Quality Assessment of Herbal Raw Material

4.2.1. Botanical Evaluation

4.2.1.1. Macroscopic characteristics

The macroscopic characteristics of the selected herbal samples are presented in Table 9. The images of the selected herbal raw materials are depicted in Figure 5.

Table 9: Macroscopic characteristics of herbs

Parameters	*G. sylvestre*	*T. foenum graecum*	*C. longa*	*P. nigrum*
Part used	Dried leaves	seeds	rhizomes	Fruits
Shape	Leaf simple, elliptical or ovate in shape	oblong	cylindrical and short branched	small round and wrinkled
size	3-6cm long and 1-3cm broad	0.2-0.5 cm long, 0.15-0.35 cm broad	2-5cm long and 1-2cm thick	0.4-0.5cm in diameter
Color	Dark green to brownish green	yellow	dark yellow	greyish black to black
Odor	Unpleasant	pleasant	characteristics	aromatic
Taste	bitter and acrid	bitter	spicy	pungent

Figure 5: Selected herbal raw materials a) *G. sylvestre* leaves, b) *T. foenum-graecum* seeds, c) *C. longa* rhizomes, and d) *P. nigrum* fruits

4.2.1.2. Microscopic characteristics

Examination of microscopic characteristics is the first step towards establishing identity and purity of crud drugs. Microscopic characters observed for each herbal sample has been depicted in Figure 6-9.

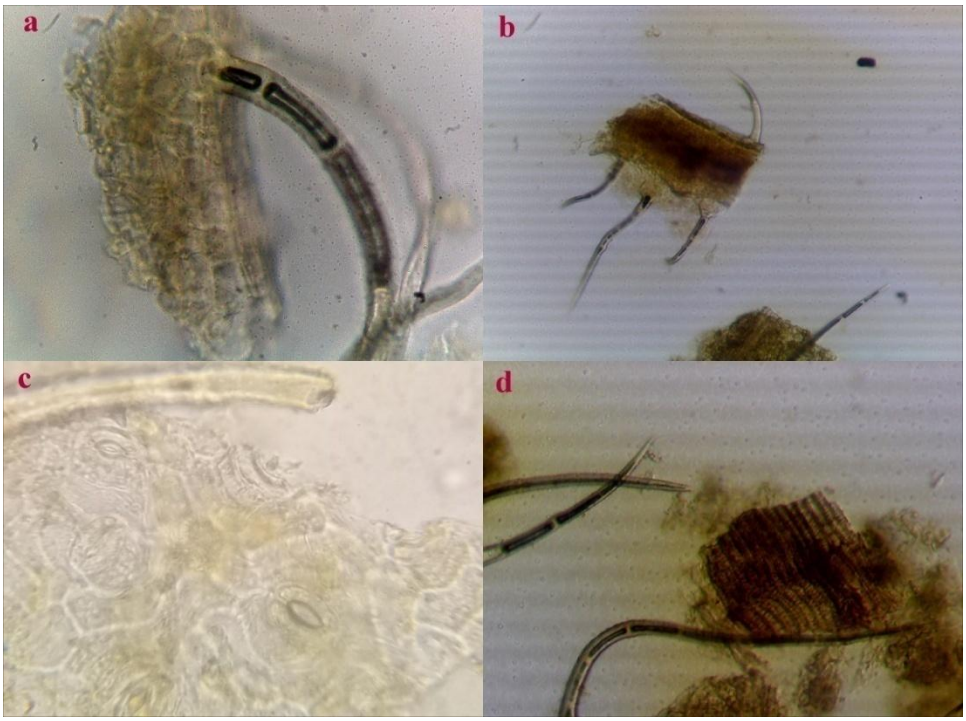


Figure 6: Microscopic characters of *Gymnema sylvestre* showing leaf, a) group of epidermal cells with multicellular trichome, b) Multicellular trichomes, c) stomata, d) group of xylem vessels

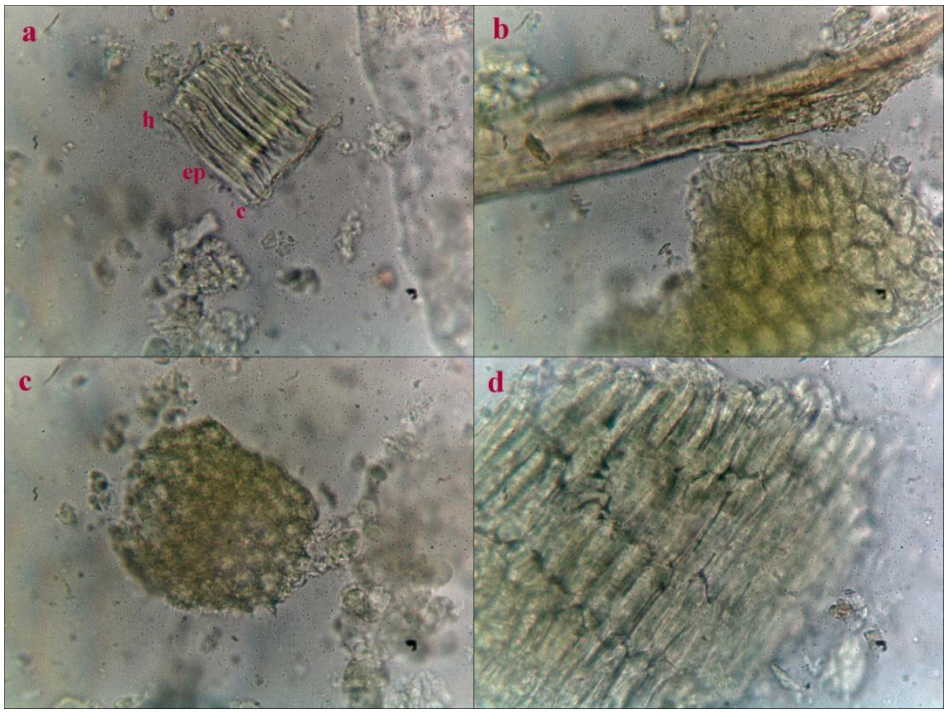


Figure 7: Microscopic characters of *Trigonella foenum-graecum* showing, a) Cuticle 'c', epidermis 'ep' and hypodermis 'h', of the testa, b)epidermis of the testa , c) hypodermis of the testa, d) parenchyma cells

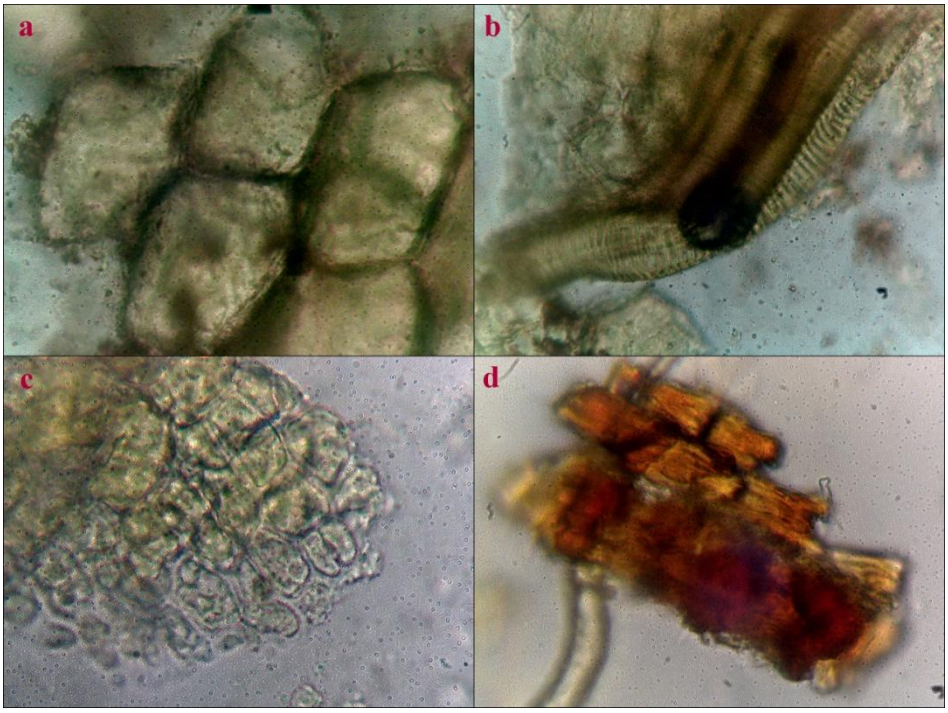


Figure 8: Microscopic characters of *Curcuma longa* showing, a) Cork cells b) thickened vessel, c) Epidermis in surface view, d) parenchymatous cells filled with yellow coloring matter

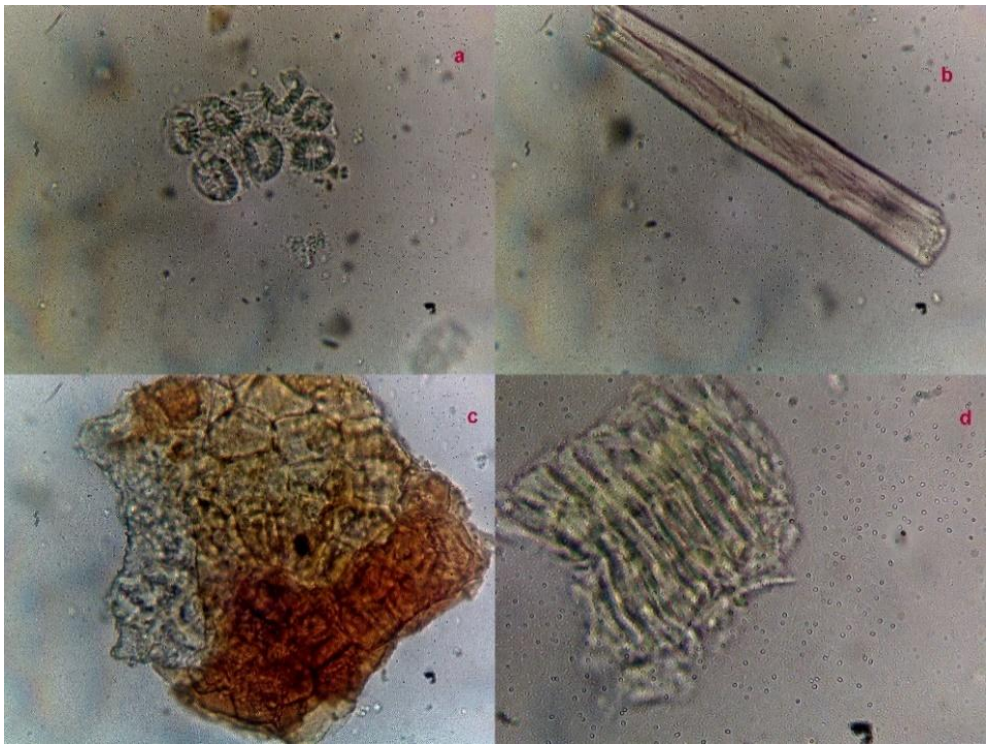


Figure 9: Microscopic characters of *Piper nigrum* showing, a) sclereids, b) Fiber, c) endocarp cells, d) group of vessels

4.2.2. Physico-chemical evaluation

Physicochemical parameters such as moisture content, extractive values, total ash value, acid insoluble ash value, and water-soluble ash value, was evaluated for each herbal raw material and were found to be within the specified limits. Table 10 summarizes the studied physico-chemical parameters.

Table 10: Physico-chemical evaluation of herbal drugs

Parameters	*G. sylvestre*	*T. foenum graecum*	*C. longa*	*P. nigrum*
Moisture content (% LOD)	7.20±0.35	7.3±0.35	6.55±0.51	7.6±0.69
Water soluble extractive value (%)	29.85±1.99	33.57±1.29	13.83±0.76	8.67±0.58
Alcohol soluble extractive value (%)	7.50±0.40	21.67±0.58	12.33±0.58	9.83±0.76
Petroleum ether soluble extractive value (%)	1.07±0.12	4.40±0.53	1.70±0.61	6.67±0.58
Total Ash value (%)	10±0.0	3.5±0.5	6.67±0.29	3.83±0.29
Acid insoluble Ash value (%)	0.67±0.29	0.45±0.03	1.0±0.5	5.0±0.0
Water soluble Ash value (%)	4.83±0.29	2.83±0.29	4.0±0.5	2.67±0.29

Results are expressed in % Mean± SD; n=3

4.2.3. Chemical evaluation

4.2.3.1. Phytochemical analysis

Preliminary phytochemical analysis was performed to identify the presence of secondary metabolites in the selected herbal raw materials. Table 11 represents the secondary metabolites present in each herbal raw material.

Table 11: Phytochemical investigation of crude drugs

Sr. No.	Phytochemicals	Test	*G. sylvestre*	*T. foenum-graecum*	*C. longa*	*P. nigrum*
1.	Alkaloids	Mayer test	+	+	+	+
		Dragendroff test	+	+	+	+
		Wagner's test	+	+	+	+
2.	Flavonoids	Shinoda test	+	+	+	+
3.	Tannins	Lead acetate	+	+	+	+
4.	Phenols	Ferric chloride	+	+	+	-
5.	saponins	Froth test	+	+	-	-
6.	Glycosides	Liebermann test	+	+	-	-
7.	Steroids	Salkawoski test	+	+	+	+

4.2.3.2. Determination of Aflatoxins

The presence of toxic substances such as aflatoxin B1, B2, G1, and G2 were analyzed in each herbal raw material and was found to be absent in all four selected herbal samples. Figure 10 depicts the chromatogram obtained for standard Aflatoxins and raw material samples.

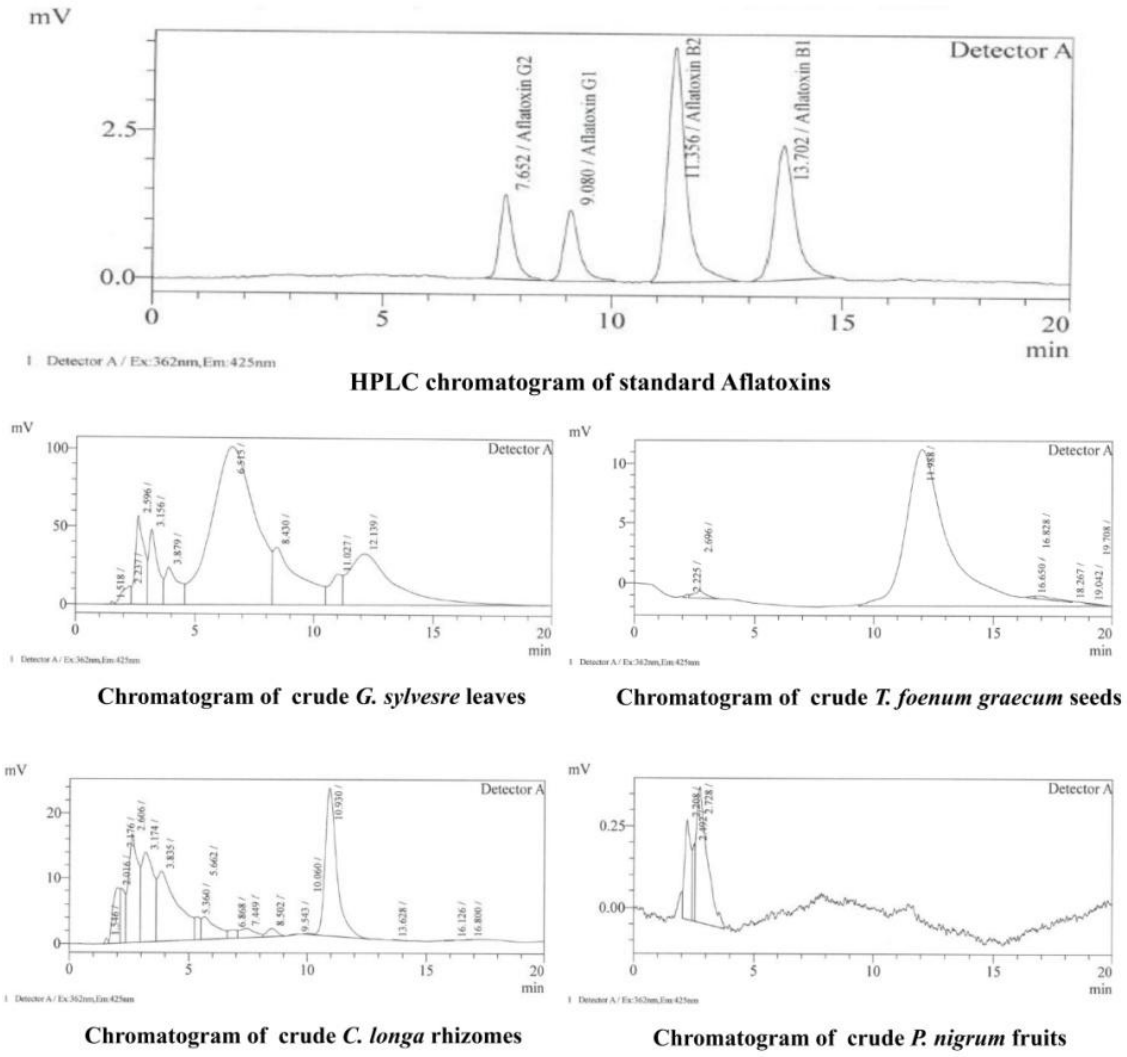


Figure 10: HPLC Chromatograms for estimation of Aflatoxins

4.2.3.3. Determination of Pesticide Residue

The presence of total 17 pesticide contaminants was determined in each herbal raw material. The results are summarized in Table 12.

Table 12: Estimation of Pesticide contaminants in Herbal raw materials

pesticides	*G. sylvestre*	*T. foenum graecum*	*C. longa*	*P. nigrum*
DDT	Below limit of Quantification	Below limit of Quantification	Below limit of Quantification	Below limit of Quantification
Lindane				
α-HCH				
β-HCH				
δ-HCH				
2, 4-dichlorophenoxyacetic acid				
Endosulphon				
Manocrotophos				
Ethion				
Chlorpyrifos				
Phorate				
Butalchlor				
Alachlor				
Atrazine				
Methyl Parathion				
Malathion				
Aldrin				

Limit of Quantification: 0.01ppm

4.2.3.4. Determination of Heavy metals

Analysis of heavy metals such as lead, cadmium, arsenic, mercury, and chromium were tested in each crude herb sample. Results demonstrated the absence of heavy metals in all the four selected herbal samples. (Table 13)

Table 13: Determination of Heavy metals in Herbal raw materials

Heavy metals	*G. sylvestre*	*T. foenum graecum*	*C. longa*	*P. nigrum*
Lead (NMT 10ppm)	Absent	Absent	Absent	Absent
Cadmium (NMT 0.3ppm)				
Mercury (NMT 10ppm)				
Arsenic (NMT 5ppm)				
Chromium (NMT 0.5 ppm)				

4.2.4. Biological Evaluation

4.2.4.1. Microbial load

Microbial load in the selected herbal crude drugs was evaluated in terms of total viable count and represented in table 14.

Table 14: Microbial load in herbal raw materials

Microbial load	*G. sylvestre*	*T. foenum graecum*	*C. longa*	*P. nigrum*
Total aerobic bacterial count (NMT 10^3 CFU/g)	300 CFU/g	250 CFU/g	150 CFU/g	200 CFU/g

4.2.5. Chromatographic Analysis

AQbD assisted HPLC method was adopted for the quantification of phytocompounds in the selected herbal raw material. Based on the evidences from the literature various trials were performed to find the suitable combination of aqueous and organic solvents to elute the phytocompounds of interest. Further, to find the optimized chromatographic condition AQbD principles were applied as follows;

4.2.5.1. Defining of Analytical Target Profile (ATP)

The ATP of the proposed analytical method is to achieve a good separation for quantification of GYM, TRG, CUR, DMC, BDC, and PRN, with lesser tailing factor and peak width along with acceptable analysis time.

4.2.5.2. Determination of Critical Quality attributes (CQA)

Based on the above mentioned ATP, CQA were identified for the development of HPLC method and are summarized in Table 15.

Table 15: CQA for optimization of HPLC method

Quality Attributes of Analytical method	Target	Justification
Tailing factor	NMT 2	Acceptance limit as per ICH guidelines
Peak width	NMT 2	Specifications as per defined ATP

4.2.5.3. Method optimization by DoE

As per the adopted 2^2 full factorial design, 4 experimental runs were designed by varying the two selected independent variables i.e., concentration of acid in aqueous phase (X1) and mobile phase ratio (X2). The responses of the dependent variables obtained for each chromatographic trial are summarized in Table 16-19.

Table 16: Selected variables and Responses for Gymnemagenin

Code	Coded levels		Actual values		Responses	
	X1	X2	X1	X2	R1	R2
T1	-1	-1	0.1%	80:20	1.19	1.17
T2	-1	+1	0.1%	85:15	1.16	1.06
T3	+1	+1	0.2%	85:15	1.25	1.21
T4	+1	-1	0.2%	80:20	1.29	1.33

X1: Conc. Of Ortho-phosphoric acid (%); X2: Mobile phase ratio; R1: Tailing factor; R2: Peak width

Table 17: Selected variables and Responses for Trigonelline

Code	Coded levels		Actual values		Responses	
	X1	X2	X1	X2	R1	R2
T1	-1	-1	0.01%	60:40	1.31	0.41
T2	-1	+1	0.01%	70:30	1.13	0.24
T3	+1	+1	0.02%	70:30	1.19	0.30
T4	+1	-1	0.02%	60:40	1.36	0.48

X1: Conc. of Hydrochloric acid (%); X2: Mobile phase ratio; R1: Tailing factor; R2: peak width

Table 18: Selected variables and Responses for Curcuminoids

Code	Coded levels		Actual values		Responses					
					Tailing factor CUR	Peak width CUR	Tailing factor DMC	Peak width DMC	Tailing factor BDC	Peak width BDC
	X1	X2	X1	X2	R1	R2	R3	R4	R5	R6
T1	-1	+1	0.02%	60:40	1.37	1.23	1.37	0.85	1.41	0.69
T2	-1	-1	0.02%	55:45	1.25	0.97	1.27	0.74	1.29	0.6
T3	+1	-1	0.05%	55:45	1.29	0.91	1.31	0.8	1.37	0.63
T4	+1	+1	0.05%	60:40	1.42	1.15	1.42	0.9	1.48	0.73

X1: Conc. of Ortho-phosphoric (%); X2: Mobile phase ratio; R1: Tailing factor CUR; R2: peak width CUR; R3: Tailing factor DMC; R4: peak width DMC; R5: Tailing factor BDC; and R6: peak width BDC

Table 19: Selected variables and Responses for Piperine

Code	Coded levels		Actual values		Responses	
	X1	X2	X1	X2	R1	R2
T1	-1	-1	0.05%	60:40	1.31	0.49
T2	-1	+1	0.05%	70:30	1.23	0.43
T3	+1	+1	1 %	70:30	1.47	0.55
T4	+1	-1	1 %	60:40	1.57	0.62

X1: Conc. of Acetic acid (%); X2: Mobile phase ratio; R1: Tailing factor; R2: peak width

Statistical optimization of the analytical methods was performed by comparison of several statistical parameters, provided by Design-Expert® Software, Version 12. The statistical data for each compound i.e. GYM, TRG, CUR, DMC, BDC, and PRN are summarized in Table 20.

Table 20: Summary of statistical parameters and polynomial equation

Response		P-value	Model Significance	Polynomial equation
GYM	R1	0.0493	Significant	+1.22+0.0475*X1-0.0175*X2
	R2	0.0259	Significant	+1.19+0.0775*X1-0.0575*X2
TRG	R1	0.0272	Significant	+1.2+-0.0275*X1-0.0875*X2
	R2	0.0268	Significant	+0.3575+0.0325* X1-0.0875*X2
CUR	R1	0.0376	Significant	+1.33+0.0225* X1 -0.0625X2
	R2	0.0385	Significant	+1.07-0.0350* X1-0.1250*X2
DMC	R3	0.0437	Significant	+1.34+0.0225*X1-0.0525*X2
	R4	0.0421	Significant	+0.8225+0.0275*X1-0.0525*X2
BDC	R5	0.0364	Significant	+1.39+0.0375*X1-0.0575X2
	R6	0.0493	Significant	+0.6625+0.0175*X1-0.0475*X2
PRN	R1	0.0376	Significant	+1.39+0.1250* X1-0.0450*X2
	R2	0.0355	Significant	+0.5225+0.0625*X1-0.0325*X2

The ANOVA performed for studying the effect of independent variables on dependent variables showed statistical significance for all the studied responses at p-value < 0.05. In the derived polynomial equations representing the relationship between the independent variables (X) and responses (R), a positive sign of the coefficient indicates a synergistic effect whereas, a negative term indicates an antagonistic effect upon the responses. The greater coefficient means that the independent variable has a

more potent influence on the response. The effect of each factor on responses was demonstrated graphically in terms of 3D-Response Surface Plots. (Figure 11-14).

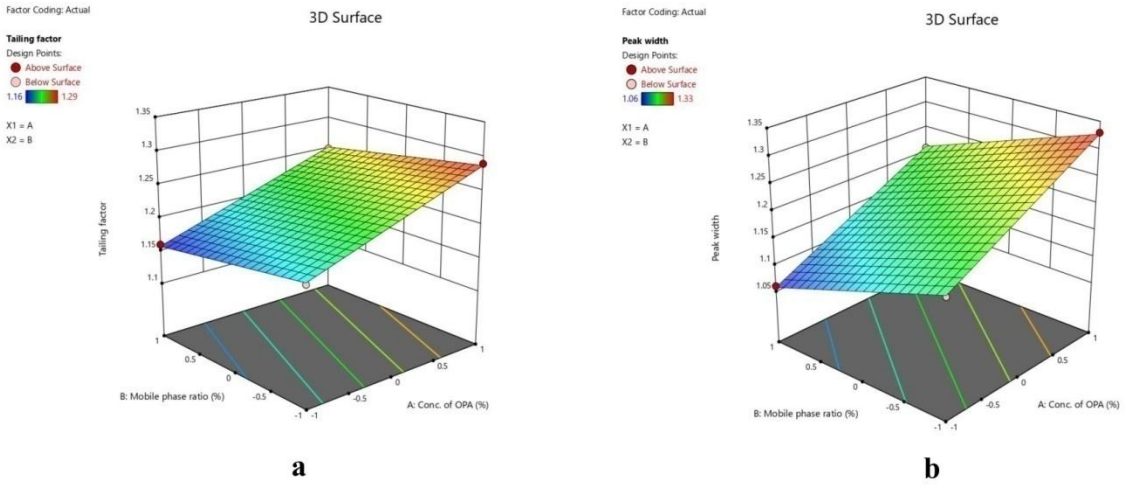


Figure 11: 3-D response surface plot a) tailing factor (R1), b) Peak width (R2) for optimization of HPLC method for Gymnemagenin

Interpretation: From the Response Surface plot, it can be observed that, as the concentration of orthophosphoric acid decreases, the tailing factor and peak width also decreases. Further, as the ratio of methanol in the mobile phase increases peak width decreases. Moreover, it can be seen that the tailing factor is not much affected by the change in the ratio of the mobile phase.

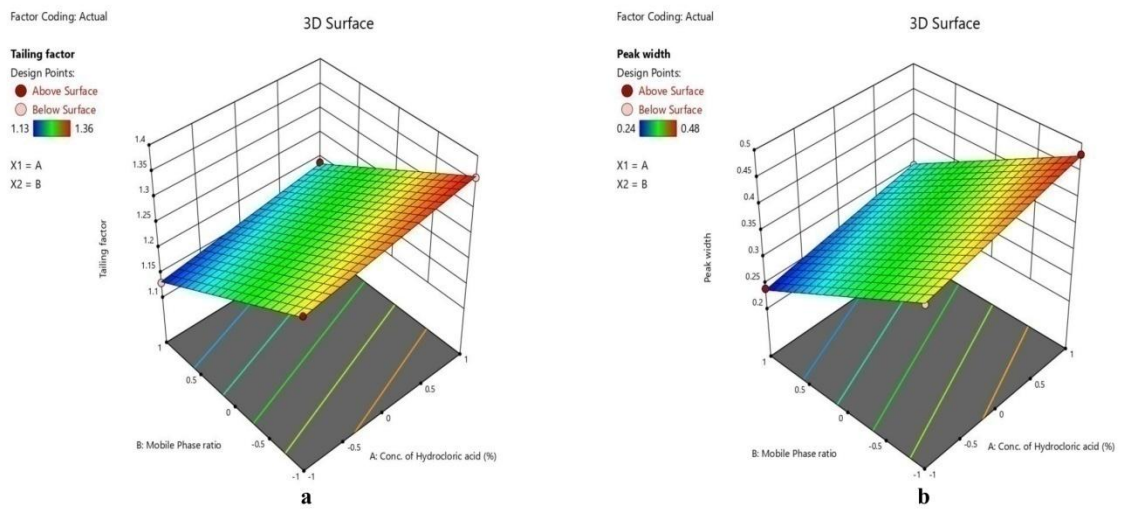


Figure 12: 3-D response surface plot a) tailing factor (R1), b) Peak width (R2) for optimization of HPLC method for Trigonelline

Interpretation: From the Response Surface Plot it is observed that by decreasing the level of variable X1; Concentration of Hydrochloric acid both the responses tailing factor and peak width decreases. Whereas, increasing the variable X2 i.e. Mobile phase ratio of aqueous phase the value of both the responses decreases. This indicates that variable X1 and X2 has significant impact on response R1 and R2.

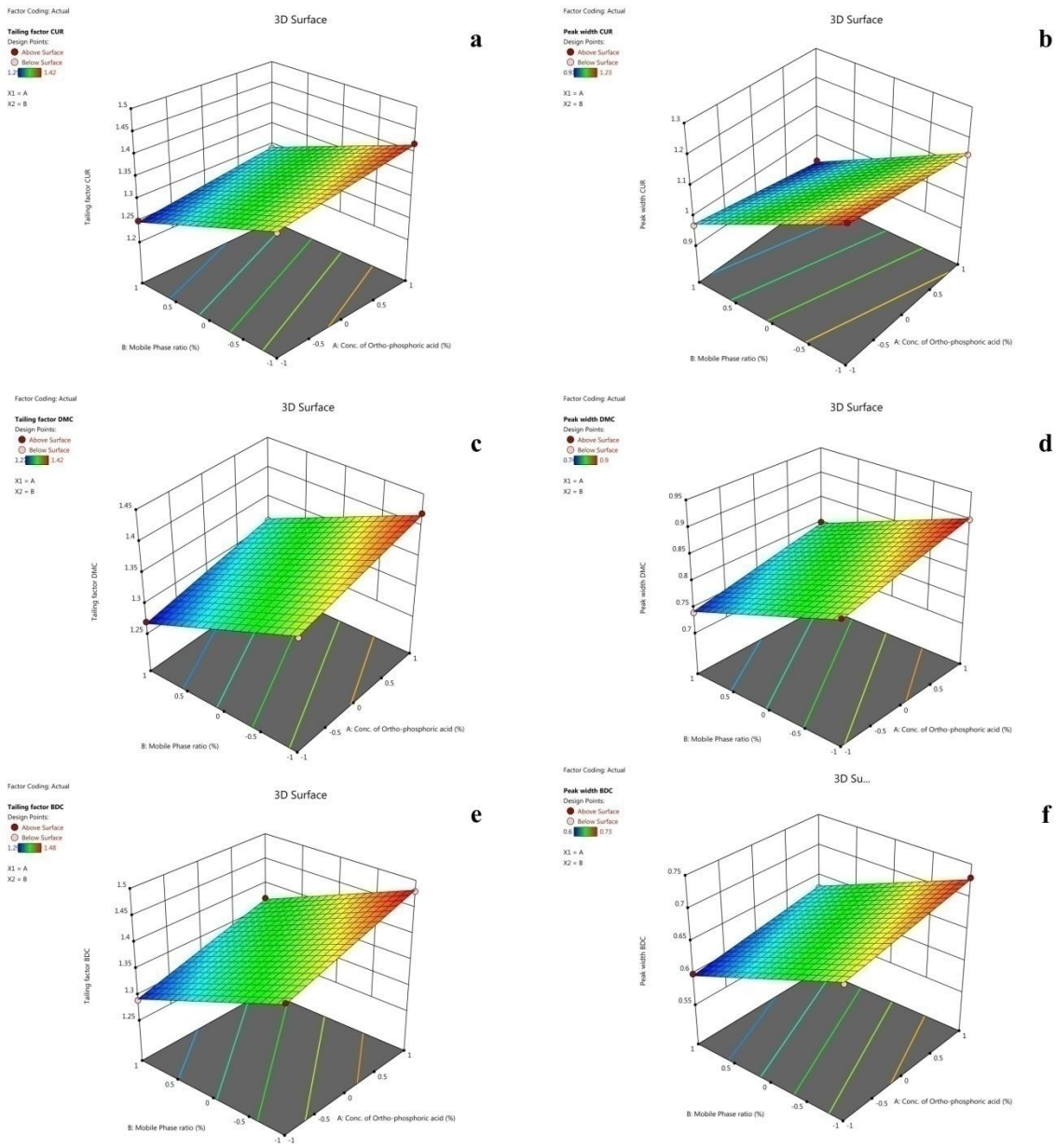


Figure 13: 3-D response surface plot a) tailing factor CUR (R1), b) Peak width CUR (R2) c)Tailing factor DMC (R3), d) peak width DMC (R4), e) tailing factor BDC (R5), and f) peak width BDC (R6) for optimization of HPLC method for Curcuminoids

Interpretation: From the Response Surface Plot it is evident that by decreasing the concentration of orthophosphoric acid (X1), tailing factor (R1, R3, & R5) and peak width (R2, R4 & R6) decreases.

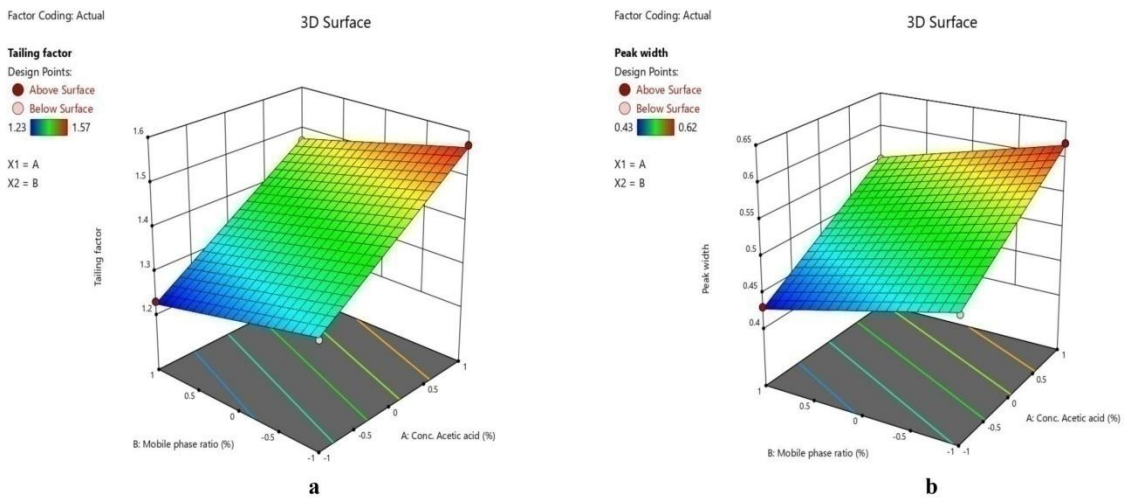


Figure 14: 3-D response surface plot a) tailing factor (R1), b) Peak width (R2)for optimization of HPLC method for Piperine

Interpretation: From the plot it is observed that by decreasing the level of variable X1 i.e. Concentration of acetic acid in the aqueous phase, the value of both the responses R1and R2 decreases which means that by decreasing concentration of acetic acid in aqueous phase tailing factor and peak width decreases. This further indicates that variable X1 has a significant impact on response R1 and R2.

4.2.5.4. Establishment of MODR

MODR is a multidimensional combination and interaction of independent factors which, further lead to the selection of acceptable operating ranges that assure quality. Figure 15-18 shows MODR (overlay plot) with the optimum region as a design space in yellow shade and selected method conditions represented using a flag. The optimized chromatographic conditions for the selected phytoconstituents are summarized in table 21.

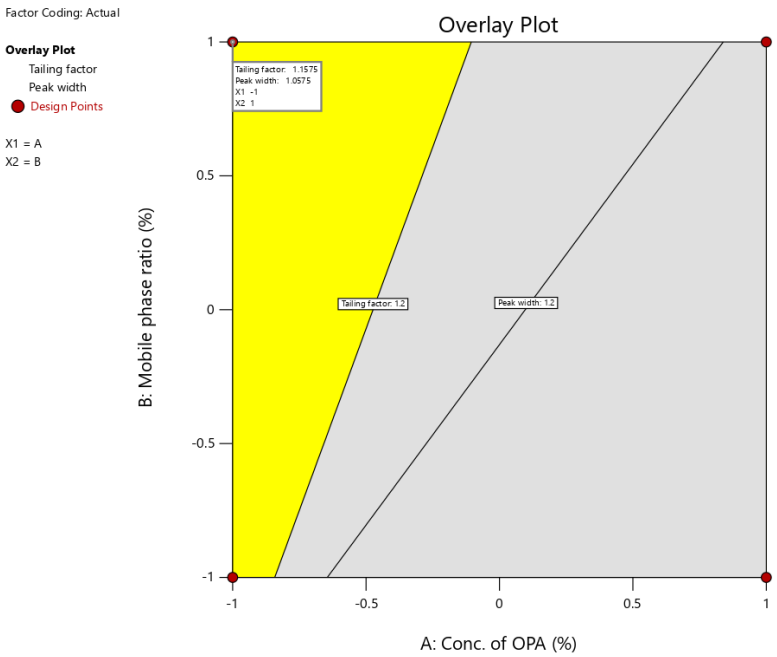


Figure 15: MODR for optimization of HPLC method for Gymnemagenin

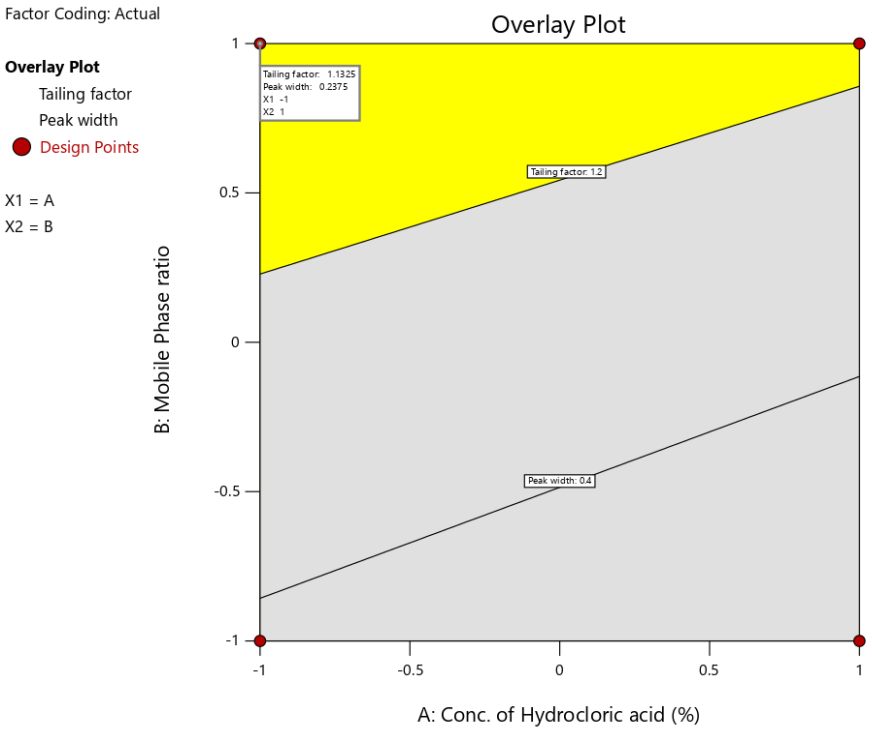


Figure 16: MODR for optimization of HPLC method for Trigonelline

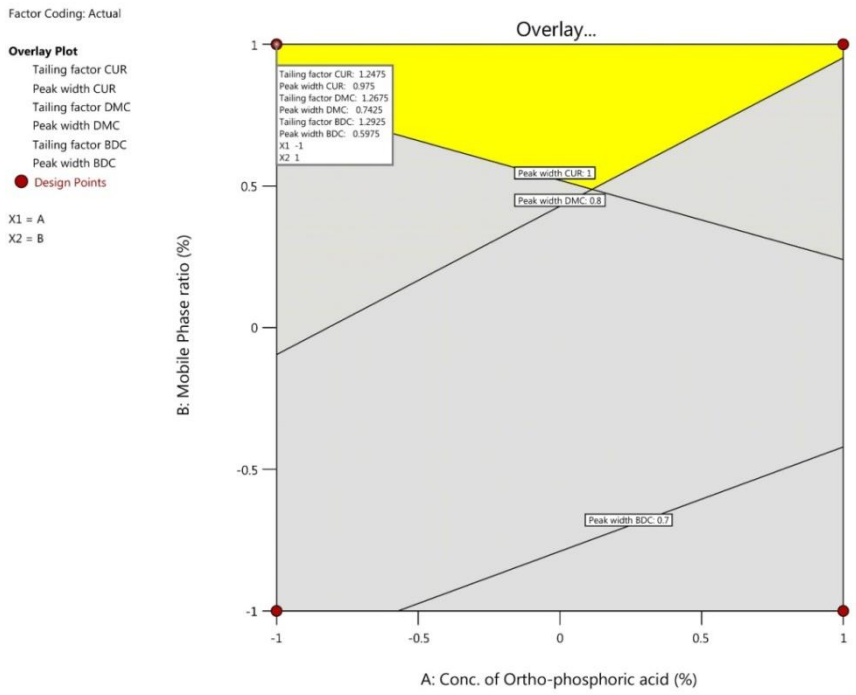


Figure 17: MODR for optimization of HPLC method for Curcuminoids

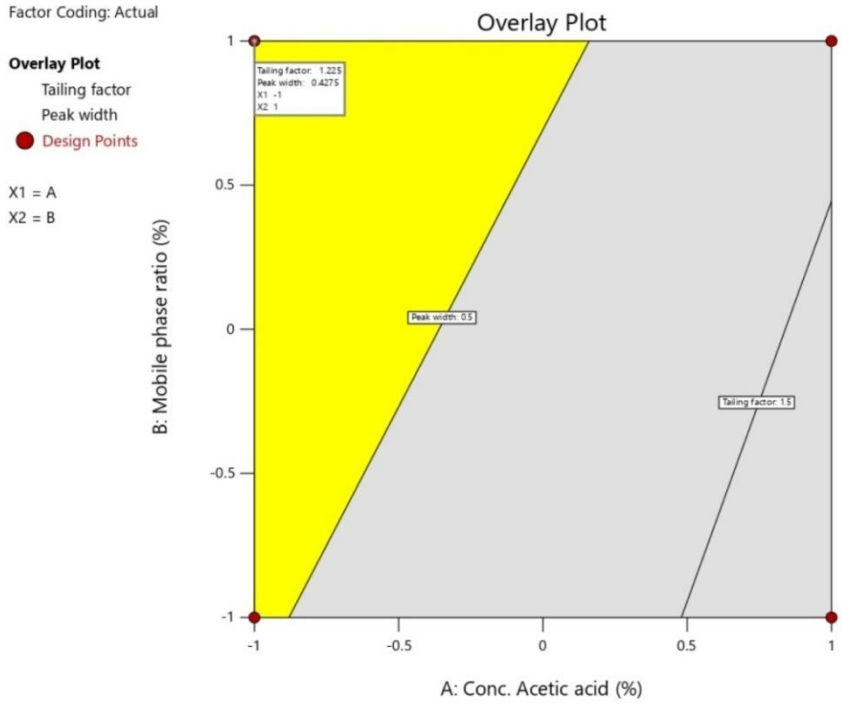


Figure 18: MODR for optimization of HPLC method for Piperine

Table 21: Optimized chromatographic condition

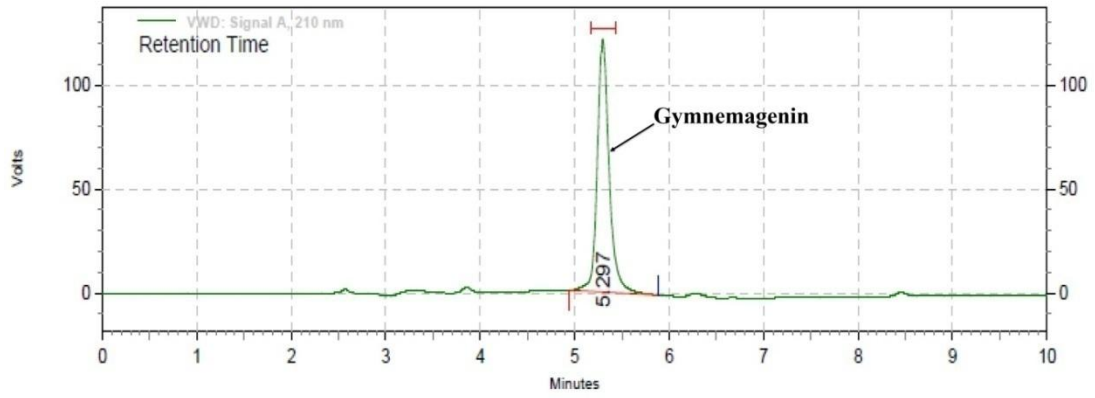
Parameters	Chromatographic conditions			
	Gymnemagenin	**Trigonelline**	**Curcuminoids**	**Piperine**
Stationary Phase	ZORBAX C-18 (250mm x 4.6 mm, 5µ) column			
Mobile phase	Methanol: Water (0.1% OPA)	Water (0.01% HCl): Methanol	Acetonitrile: Water (0.02% OPA)	Acetonitrile: Water (0.05% Acetic acid)
Ratio	85:15	70:30	55:45	70:30
Flow rate	0.8 ml/min	1ml/min	1ml/min	1ml/min
Detection wavelength	210 nm	263nm	425nm	342 nm
Retention time	5.29 min	2.877 min	CUR-9.86 min, DMC-9.11 min, BDC- 8.44 min	5.54 min

4.2.5.5. Quantitative estimation of Phytocompounds

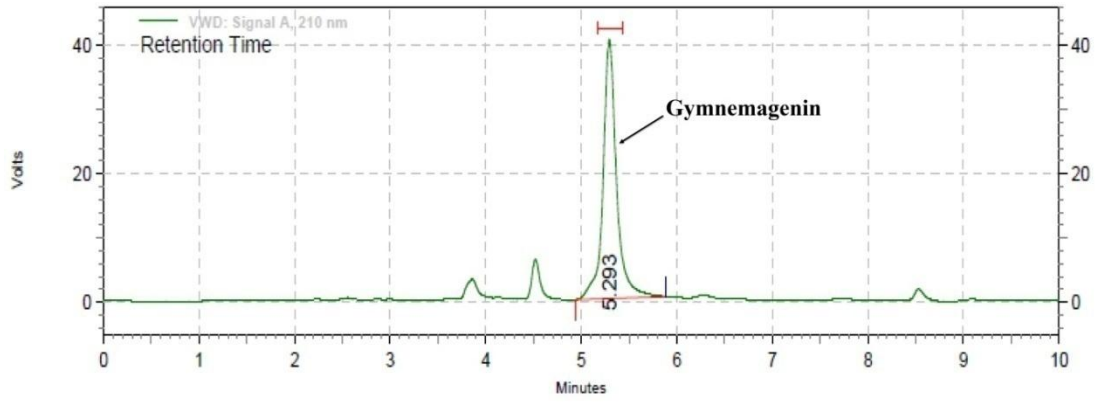
The optimized methods were further applied for the quantitative estimation of Gymnemagenin, Trigonelline, Curcuminoids, and Piperine in crude samples of *G. sylvestre*, *T. foenum graecum*, *C. longa* and *P. nigrum* respectively. Table 22 summarizes the quantity of each phytocompound estimated from the respective plant material. The chromatograms obtained for each standard marker and samples are depicted in Figure 19-22.

Table 22: HPLC quantification data for selected phytocompounds

Phytocompounds/ Marker compound		Raw material taken (mg)	Amount of marker obtained (mg)	Content of marker (% w/w)
GYM		500	25.54	5.11±0.002
TRG		1000	5.837	0.58±0.001
Curcuminoids	BDC	1000	93.24	9.32±0.20
	DMC	1000	109.84	10.98±0.23
	CUR	1000	138.62	13.86±0.28
PRN		1000	36.26	3.63±0.06

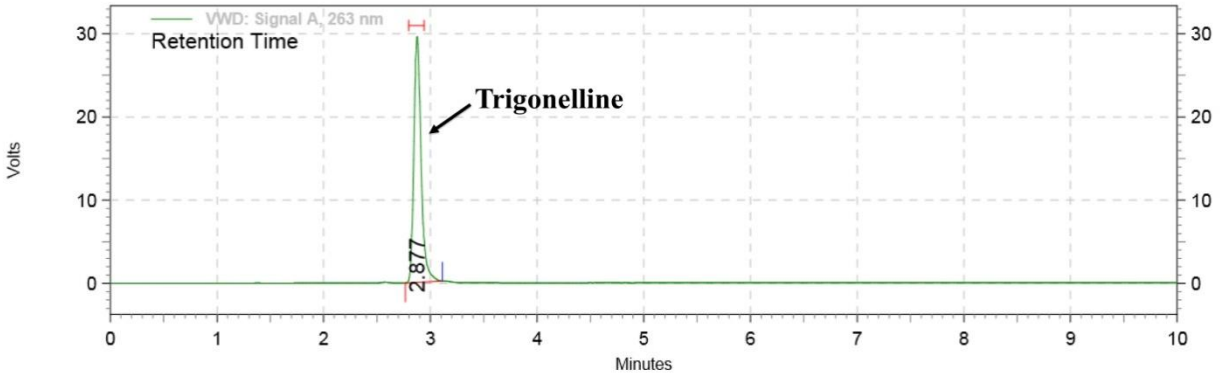


HPLC Chromatogram of standard Gymnemagenin

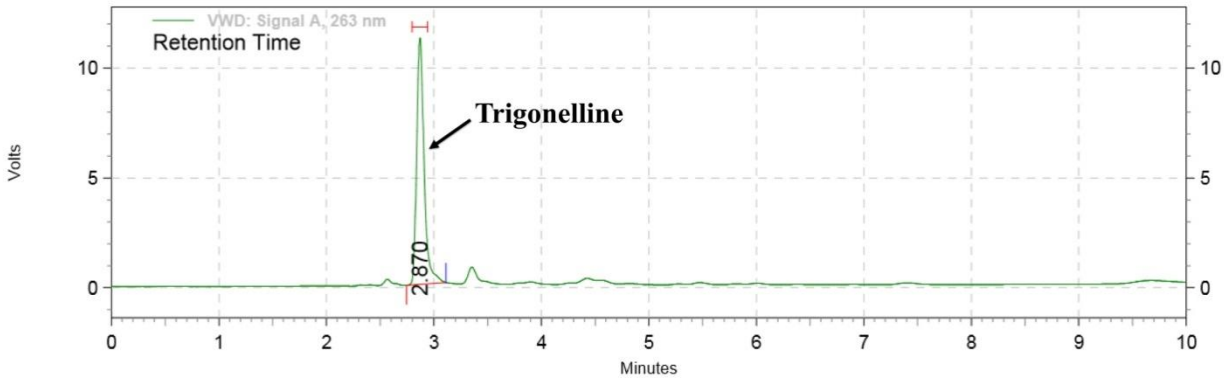


HPLC Chromatogram of Gymnemagenin in crude *G. sylvestre* leaves

Figure 19: HPLC chromatogram of Standard Gymnemagenin and crude *G. sylvestre* sample

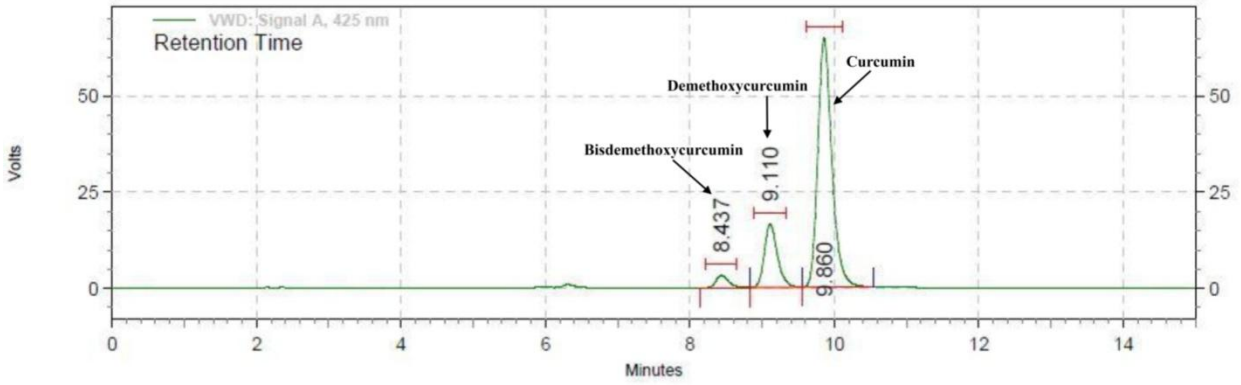


HPLC chromatogram of standard Trigonelline

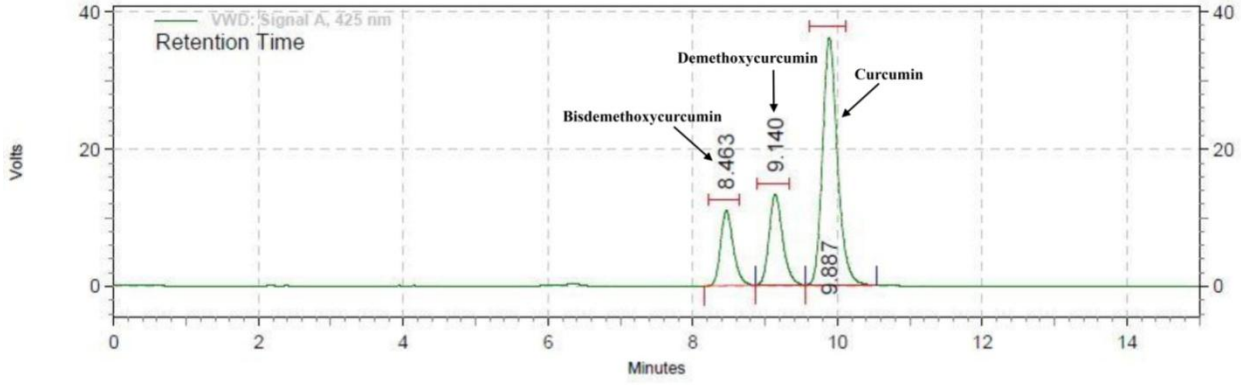


HPLC chromatogram of Trigonelline in crude *T. foenum graecum* seeds sample

Figure 20: HPLC chromatograms of standard Trigonelline and *T. foenum graecum* L. seed

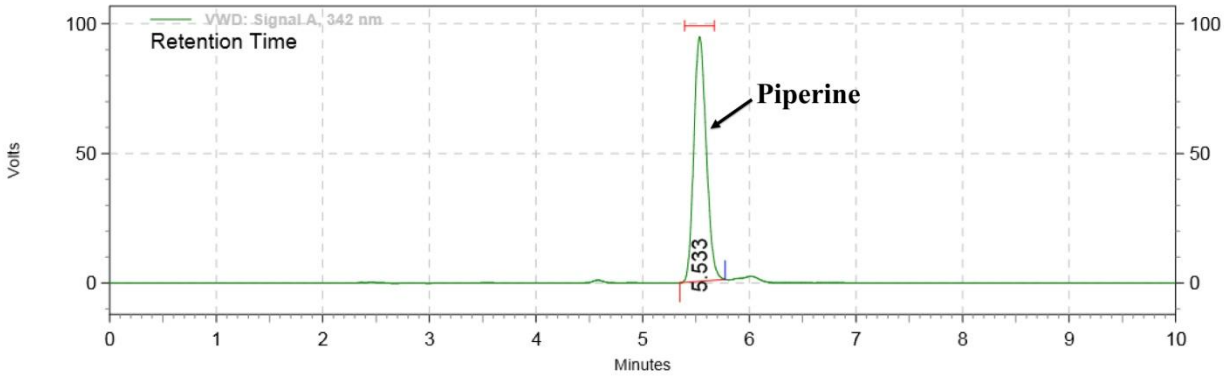


HPLC Chromatogram of Standard Curcuminoids

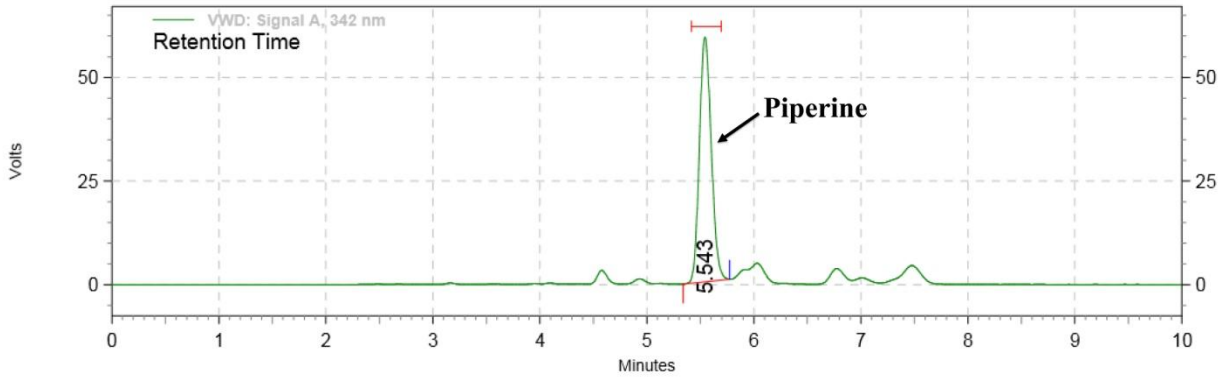


HPLC Chromatogram of Curcuminoids in crude *C. longa* rhizome sample

Figure 21: HPLC chromatograms for standard Curcuminoids and curcuminoids in *C. longa* crude sample



HPLC chromatogram of standard Piperine



HPLC chromatogram of Piperine in crude *P. nigrum* fruits sample

Figure 22: HPLC chromatograms of standard Piperine and crude *P. nigrum* L.

4.2.5.6. Method Validation

Method validation data for the developed RP-HPLC method is summarized in Table 23 & 24.

Table 23: Summary of validation parameters for GYM, TRG and PRN

Validation parameters		**GYM**	**TRG**	**PRN**
System suitability				
Retention time	Mean± SD	5.294±0.004	2.878±0.002	5.54±0.003
	% RSD	0.081	0.099	0.05
Peak area	Mean± SD	14372370±234604	2048988±28239	20156202±120229
	% RSD	1.63	1.37	0.59
Tailing factor	Mean± SD	1.17±0.02	1.32±0.008	1.24±0.01
	% RSD	1.32	0.61	0.68
Linearity				
Linearity range (µg/ml)		100-500	2-10	5-25
Correlation-coefficient		0.9989	0.9981	0.9986
LOD (µg/ml)		22.96	0.58	1.12
LOQ (µg/ml)		69.57	1.77	3.41
Precision				
Intra-Day (%RSD)		1.17	1.56	1.35
Inter-Day (%RSD)		1.70	1.66	1.56
Accuracy				
80%	% Recovery	98.79±0.44	96.93±0.35	99.85±0.28
100%	% Recovery	100.69±0.82	106.83±0.75	101.20±0.02
120%	% Recovery	102.83±0.48	110.21±0.40	96.35±0.05

Table 24: Summary of validation parameters for CUR, DMC and BDC

Validation parameters		CUR	DMC	BDC
System suitability				
Retention time	Mean± SD	9.86±0.021	9.114±0.016	8.44±0.01
	% RSD	0.22	0.17	0.15
Peak area	Mean± SD	6762417±23294	1384120±12093	254234±1189
	% RSD	0.34	0.87	0.47
Tailing factor	Mean± SD	1.31±0.010	1.30±0.01	1.33±0.01
	% RSD	0.79	0.63	0.57
Linearity				
Linearity range (µg/ml)		2-10	2-10	2-10
Correlation-coefficient		0.9985	0.9985	0.9981
LOD (µg/ml)		0.45	0.45	0.52
LOQ (µg/ml)		1.38	1.38	1.58
Precision				
Intra-Day (%RSD)		1.38	1.36	1.28
Inter-Day (%RSD)		1.60	1.58	1.35
Accuracy				
50%	% Recovery	105.21±0.34	102.13±0.46	99.88±0.49
100%	% Recovery	105.46±0.43	102.20±0.26	99.15±0.47
150%	% Recovery	101.06±0.34	98.48±0.28	95.92±0.12

4.3. Extraction

The extraction of *G. sylvestre* leaves, *T. foenum-graecum* seeds, *C. longa* rhizomes and *P. nigrum* fruits were carried out as per described procedure and the percentage yield of the each extract were calculated based on the weight of air-dried plant material. Table 25 summarizes the percentage yield of extracts obtained from each selected crude drug.

Table 25: Percentage yield of extracts

Crude drug	Percentage yield (%)
G. sylvestre	25.70
T. foenum graecum	26.92
C. longa	23.02
P. nigrum	12.52

4.4. Quality Assessment of Herbal Drug Substance (Extract)

4.4.1. Physico-chemical and flow properties

Quality assessment of the herbal drug substance i.e. plant extracts were carried out by evaluation of moisture content, powder flow properties such as; angle of repose, bulk density and tapped density. The results are as depicted in Table 26.

Table 26: Physico-chemical and Flow properties of extracts

Parameters	*G. sylvestre* extract	*T. foenum-graecum* extract	*C. longa* extract	*P. nigrum* extract
Moisture content (%)	1.97±0.20	1.48±0.08	1.02±0.08	2.18±0.21
Angle of repose (º)	32.11±0.19	29.82±0.27	29.36±0.27	32.22±0.19
Bulk density (g/cm^3)	0.456±0.002	0.591±0.004	0.498±0.003	0.5±0.00
Tapped density (g/cm^3)	0.549±0.006	0.714±0.010	0.620±0.009	0.593±0.004

4.4.2. Phytochemical analysis

Each plant extract was investigated for the presence of secondary metabolite by performing phytochemical test. The results of the same are presented in table 27.

Table 27: Phytochemical investigation of extract

Sr. No	Phytochemicals	Test	*G. sylvestre extract*	*T. foenum-graecum extract*	*C. longa extract*	*P. nigrum extract*
1.	Alkaloids	Mayer test	-	+	-	+
		Dragendroff test	+	+	-	+
		Wagner's test	+	+	+	+
2.	Flavonoids	Shinoda test	+	+	+	+
3.	Tannins	Lead acetate	+	+	+	+
4.	Phenols	Ferric chloride	+	+	+	-
5.	Saponins	Froth test	+	+	-	-
6.	Glycosides	Liebermann test	+	+	-	-
7.	Triterpenes	Salkawoski test	+	+	+	+

4.4.3. Chromatographic analysis

HPLC analysis of the extracts of *G. sylvestre*, *T. foenum-graecum, C. longa* and *P. nigrum* was carried out to quantify the active phytocompounds (marker) present in them after extraction. The quantitative estimation of GYM, TRG, CUR, DMC, BDC and PRN was carried out and the results are presented in table 28.

Table 28: Quantitative estimation of phytocompounds in extract

Plant extract / marker compounds		Content of marker compound (% w/w)
G. sylvestre (GYM)		6.23±0.37
T. foenum-graecum (TRG)		2.14±0.007
C. longa	CUR	27.44±2.25
	DMC	22.01±1.78
	BDC	18.34±1.54
P. nigrum (PRN)		5.62±0.32

4.4.4. *In-silico* molecular docking

The binding affinity and hydrogen bond interaction of each compound with the target moiety i.e. α-glucosidase and α-amylase is presented in table 29 and 30 respectively. Similarly, the interaction of the selected compounds with α-glucosidase and α-amylase is depicted in figure 23 and 24 respectively

Table 29: α-glucosidase binding affinity of Phytocompounds

Ligand	Binding Affinity (kcal/mol)	Hydrogen bond interactions	Hydrogen bond residues
Acarbose	-8.3	6	Trp 1148, Thr 1150, His1449,Leu 1450, Arg 1453, Asp1454
Gymnemagenin	-8.3	1	Thr1150
Trigonelline	-5.8	2	Trp1749, Ile 1716
Curcumin	-8.8	2	Thr1586, Asp 1279
Demethoxycurcumin	-8.1	2	Trp1369, Arg1377
Bisdemethoxycurcumin	-8.7	2	His1584, Thr 1586
Piperine	-8.7	1	Arg 1510

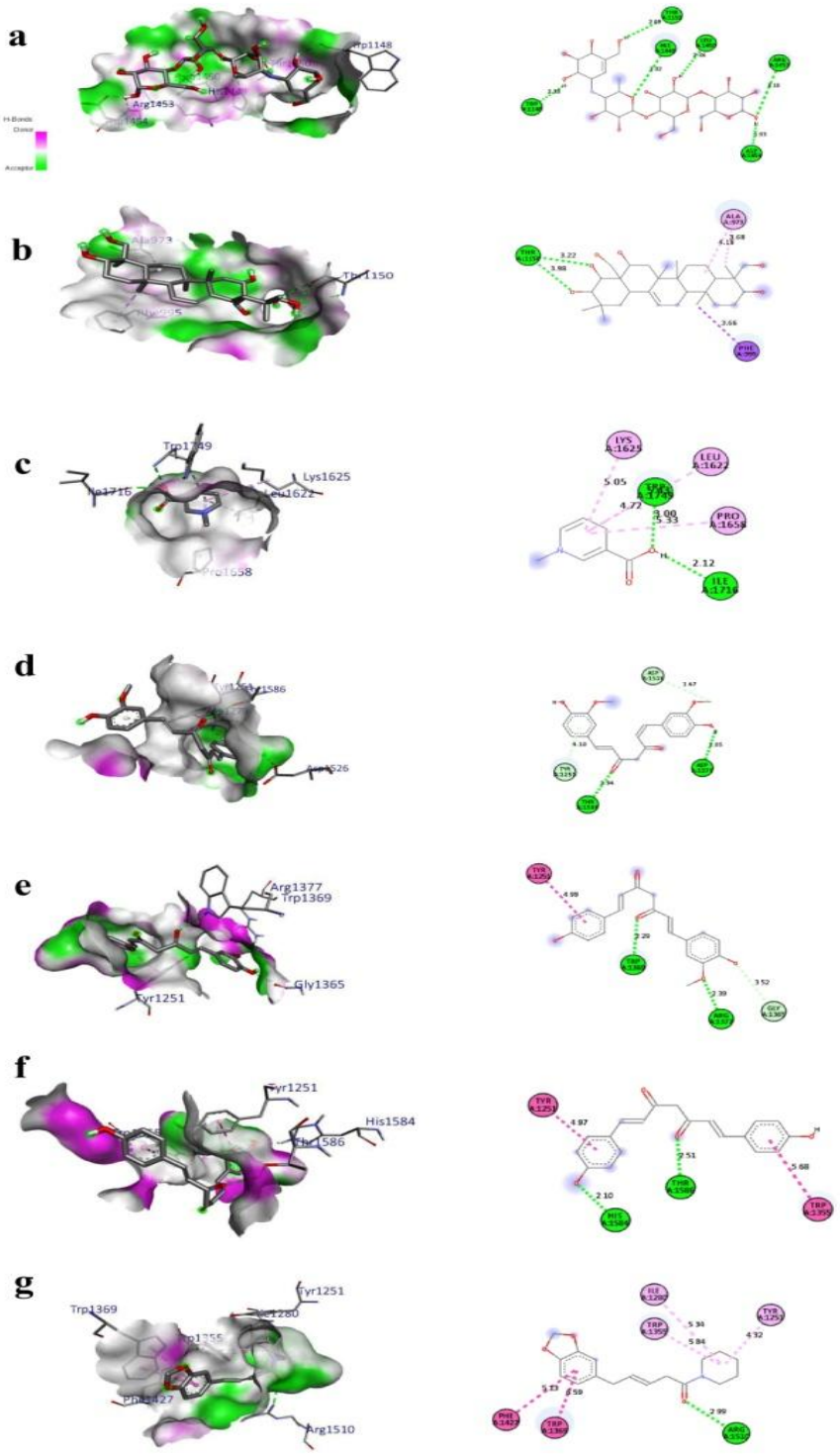


Figure 23: 3D and 2D interactions of a) Acarbose , b) Gymnemagenin, c) Trigonelline, d) Curcumin, e) Demethoxycurcumin, f) Bis-demethoxycurcumin, and g) Piperine with α-glucosidase enzyme

Docking results of α-amylase

Table 30: α-amylase binding affinity of Phytocompounds

Ligand	Binding Affinity (kcal/mol)	Hydrogen bonds	Hydrogen bond residues
Acarbose	-8.2	5	His331, Asn279, Arg421, ly334, Ser289
Gymnemagenin	-8.6	4	Thr163, Glu233
Trigonelline	-5.3	3	Asp197, Glu233, Arg195
Curcumin	-8.4	4	Asn197, Gln63, Asp356
Demethoxycurcumin	-7.6	1	Arg195
Bisdemethoxycurcumin	-8.5	4	Glu233, Gln63, Asp356
Piperine	-7.8	1	Ile235

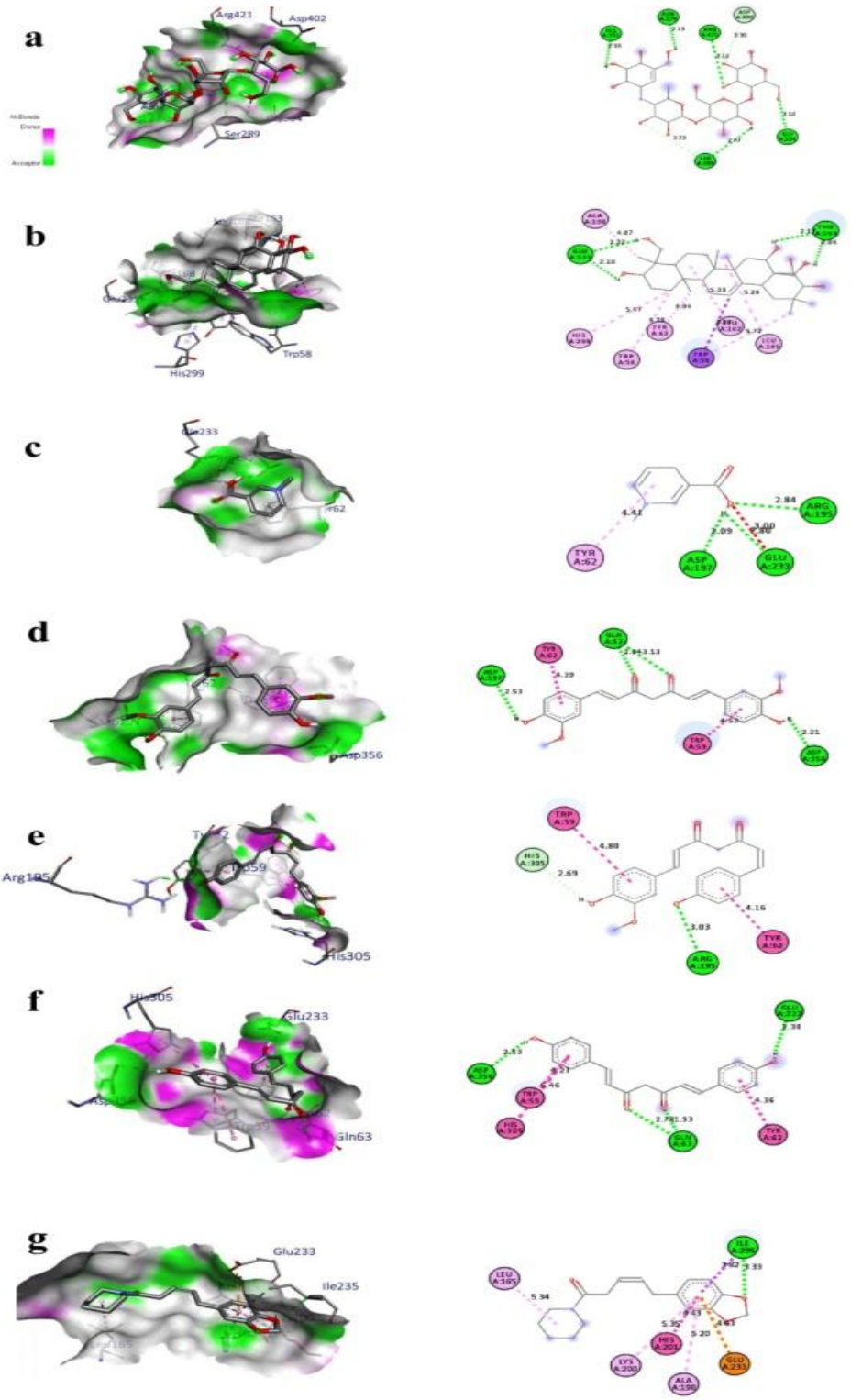


Figure 24: 3D and 2D interactions of a) Acarbose , b) Gymnemagenin, c) Trigonelline, d) Curcumin, e) demethoxycurcumin, f) bis-demethoxycurcumin, and g) Piperine with α-amylase enzyme

4.5. FORMULATION DEVELOPMENT

4.5.1. Compatibility study

4.5.1.1. FT-IR spectroscopic analysis

The FTIR spectra of individual plant extract and mixture of extract with excipients were studied (Figure 25-29). IR spectra revealed no alteration in the functional groups of extracts after mixing with excipients indicating their compatibility. Hence, it can be said that excipients used in the formulation of polyherbal tablets are compatible with the plant extracts.

Table 31: FT-IR Spectral data

Functional group	***G. sylvestre* extract**	***T. foenum graecum* extract**	***C. longa* extract**	***P. nigrum* extract**	**Extract+ Excipients**
O-H stretch	3232.83-2816.19	3102.63-3013.90	3175.93-2850.91	-	3211.48-2879.72
C=O stretch	1736.01	1645.36	1701.22	1653.00	1693.50
C=C stretch	1596.16	1456.32	1544.96	1558.48	1519.91
C-N	-	1282.79	-	1292.31	1296.16
C-O	-	-	-	1228.66	1234.44

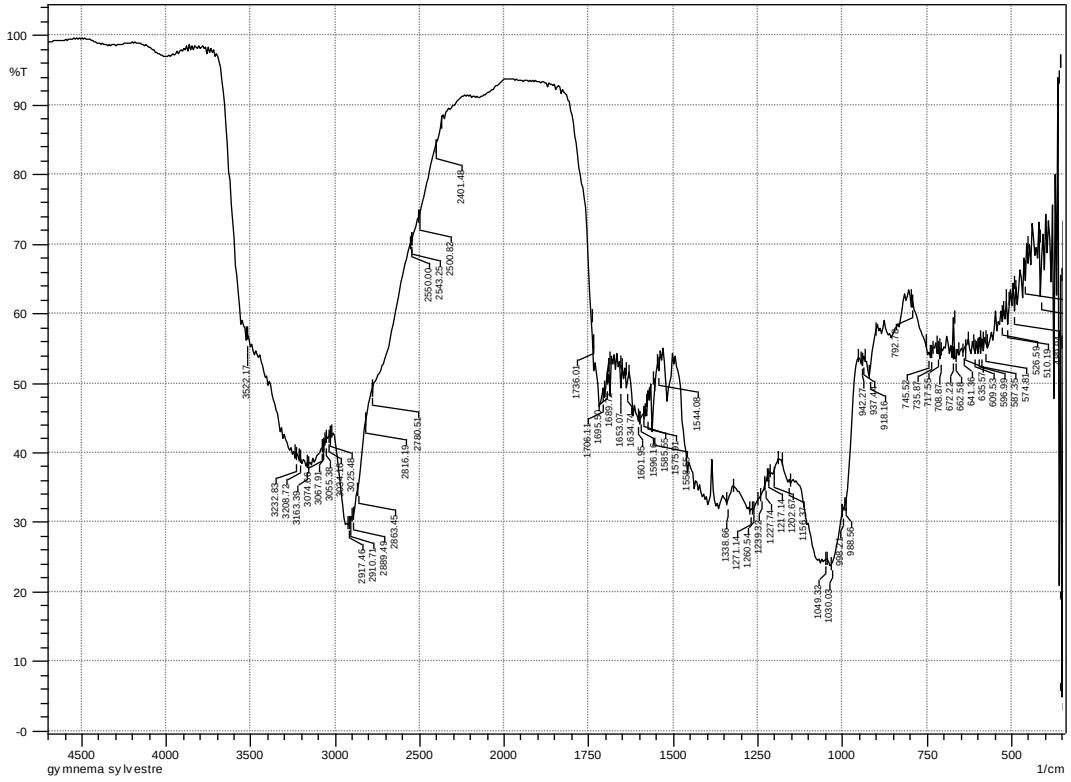


Figure 25: FT-IR spectra of *G. sylvestre* extract

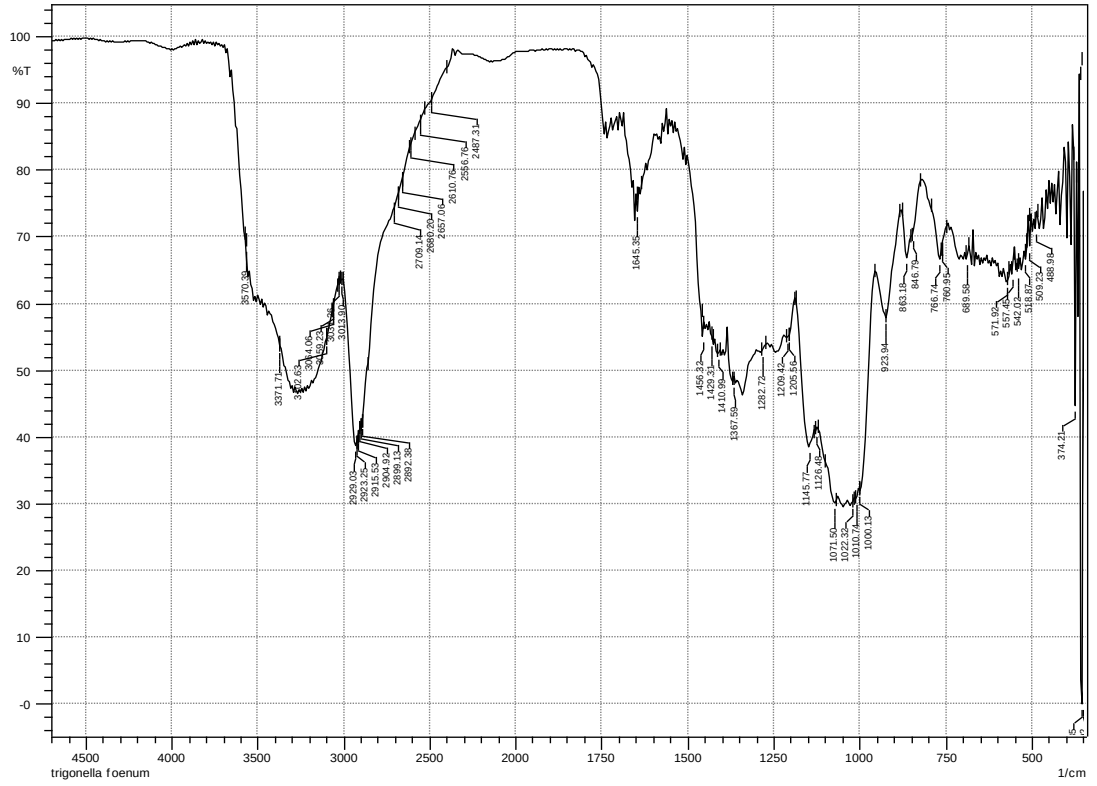


Figure 26: FT-IR spectra of *T. foenum graecum* extract

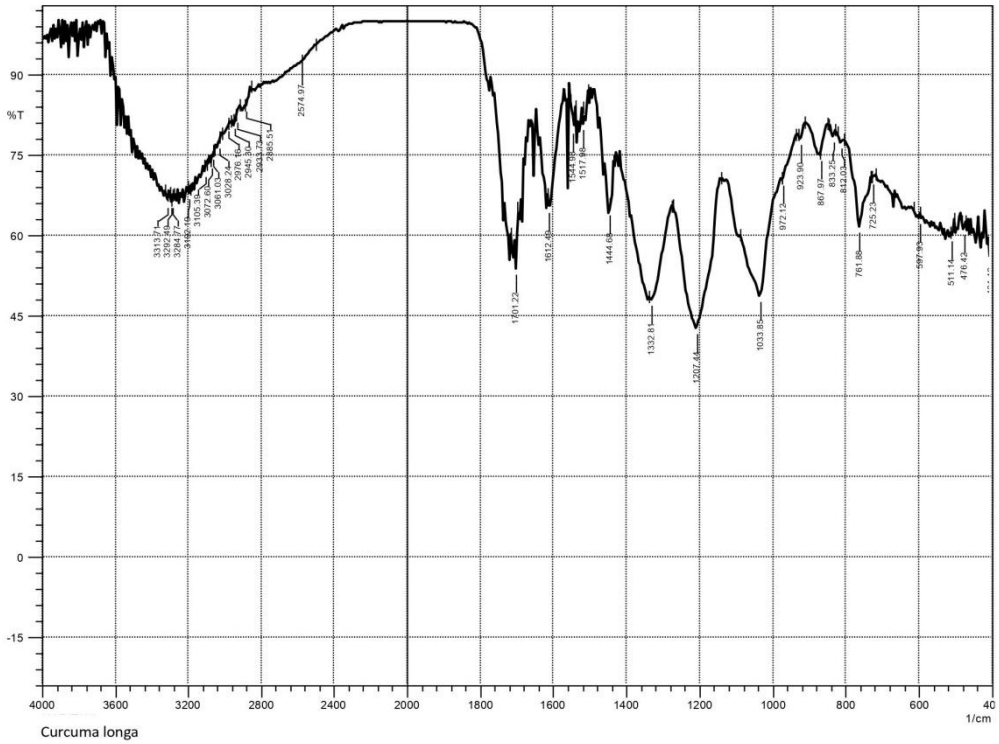


Figure 27: FT-IR spectra of *C. longa* extract

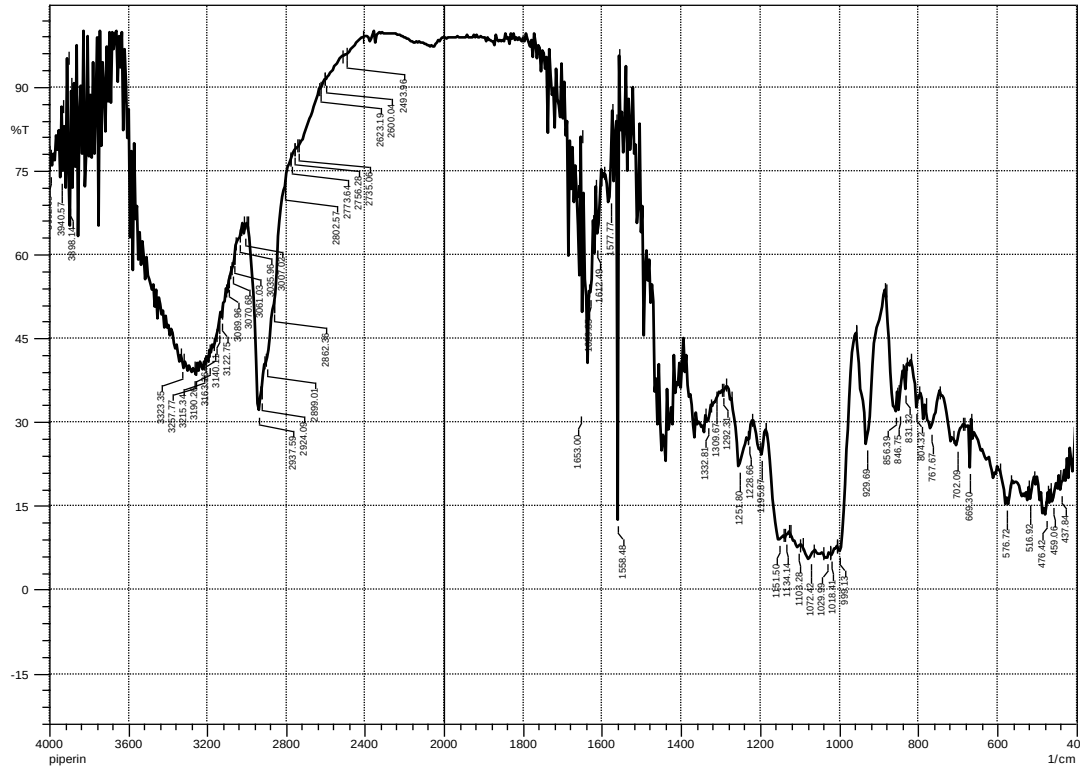


Figure 28: FT-IR spectra of *P. nigrum* extract

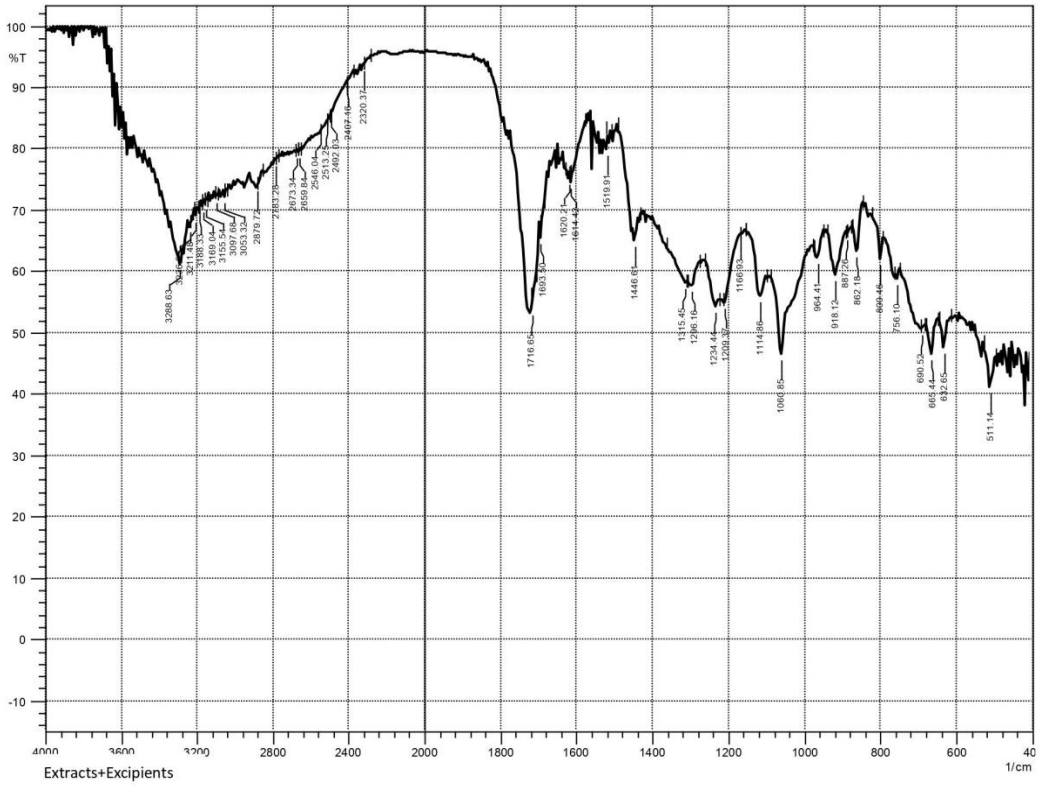


Figure 29: FT-IR spectra of Extracts+Excipients

4.5.2. Quality by Design approach for Formulation development

4.5.2.1. Development of Quality Target Product Profile (QTPP)

The QTPP for the Anti-diabetic herbal tablets have been established by taking into consideration the primary quality specification which is needed in the final product. Table 32 summarizes the QTPP for formulation of anti-diabetic herbal drug product.

Table 32: QTPP for anti-diabetic herbal drug product

QTPP Elements		Target	Justification
Therapeutic indication		Type 2 DM	-
Dosage form		Tablet	For patient acceptability
Dosage design		Conventional release tablet	patient acceptability & compliance
Route of administration		Oral	Dosage form designed to be administered orally
Dosage strength		650mg	Therapeutic dose
Drug product Quality attributes	Appearance	Tablet confirming to description, shape, and size	patient acceptability & compliance
	Disintegration time	NMT 30 min	As per product quality specification
	Hardness	3-6 kg/cm^2	
	Friability	NMT 1.0%	
Container closure system		Container closure system qualified as suitable for this drug product	Needed to achieve target shelf-life and to ensure tablet integrity during storage
Storage conditions		Store in air-tight container in a cool & dry place	To maintain tablet integrity and quality upon storage

4.5.2.2. Identification of Critical Quality Attributes (CQA)

From the Developed QTPP, CQA for the anti-diabetic formulation were defined (Table 33). The quality attributes in which the changes are likely to bring about the effect in quality of the final product and those which are considered to be critical in the formulation development point of view were considered as CQA.

Table 33: CQA for herbal anti-diabetic tablets

Quality Attributes of Drug product	Target	Justification
Disintegration time	NMT 30 min	Failure to meet the disintegration time can impact on efficacy.
Hardness	3-6 kg/cm^2	An extremely hard tablet can prevent the disintegration and dissolution of the tablet. Similarly, tablets with lesser crushing strength may lead to breakage or can give more friable tablets.
Friability	NMT 1.0%	In order to meet pharmacopoeial specification

4.5.2.3. Risk Assessment

The initial risk assessment for the formulation development of herbal anti-diabetic tablets was conducted using an Ishikawa (Fish-bone) diagram and a relative risk-ranking system. The Ishikawa diagram (Figure 30) consisted of the factors that are known to affect the quality of the herbal anti-diabetic tablets and the manufacturing process. The candidate factors which are assessed to be having greater impact on final product quality were further determined using a relative risk-ranking system (Table 34). Here, each factor was related with the CQAs and relative risk on the final product quality was predicted in terms of high, medium, or low risk.

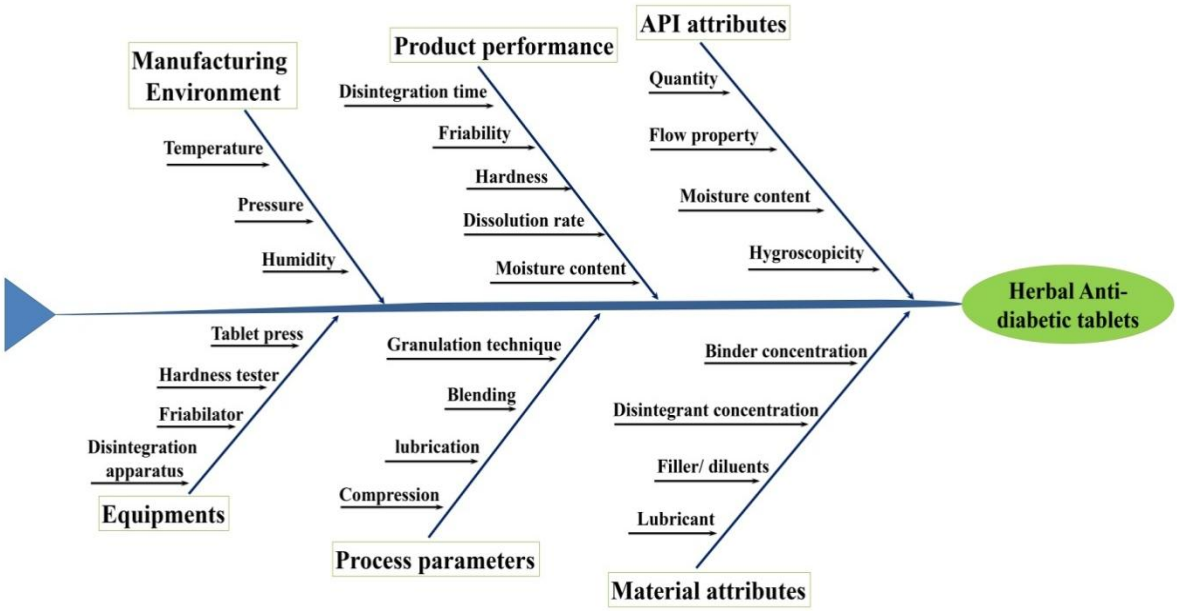


Figure 30: Ishikawa/Fish-bone diagram depicting potential factors for development of Herbal anti-diabetic tablet formulation

Table 34: Risk Assessment by relative risk-ranking system

CQA	API Attributes			Material attributes			
	Quantity	Flow properties	Moisture	Conc. of MCC	Conc. of CCS	Conc. of Anhydrous lactose	Conc. of Magnesium stearate/ talc
Disintegration time	Medium	Low	High	High	High	Medium	Low
Hardness	Low	Low	High	High	High	Low	Low
Friability	Low	Low	High	High	High	Low	Low

Low	Broadly acceptable risk. No further investigation is needed.
Medium	Risk is acceptable. Further investigation may be needed in order to reduce the risk.
High	Risk is unacceptable. Further investigation is needed to reduce the risk.

From the initial risk assessment study, the formulation attributes which had high impact on the CQA were studied in detail and further subjected to design of experiments.

4.5.2.4. Pre-compression parameters (Micromeretics study)

The powder blend prepared for formulation of each trial batch was studied for several micrometetic properties prior to compression. The results have shown good flow characteristics for the powder blend of all formulation batches. (Table 35)

Table 35: Pre-compression parameters for the powder blend

Formulation Batch	Angle of Repose (θ)	Bulk Density (g/cm^3)	Tapped Density (g/cm^3)	Compressibility Index	Hausner's Ratio
F1	30.63±0.57	0.388±0.003	0.473±0.003	18.09±0.33	1.22±0.005
F2	31.13±0.29	0.431±0.004	0.534±0.007	19.25±0.52	1.24±0.008
F3	30.14±0.28	0.450±0.004	0.550±0.010	18.02±0.99	1.22±0.015
F4	31.31±0.60	0.452±0.005	0.562±0.000	19.57±0.83	1.24±0.013
F5	30.96±0.00	0.487±0.012	0.591±0.016	17.53±0.89	1.21±0.013
F6	31.48±0.52	0.436±0.002	0.545±0.003	20.06±0.10	1.25±0.002
F7	31.13±0.29	0.431±0.004	0.523±0.006	17.53±0.52	1.21±0.008
F8	31.84±0.81	0.449±0.006	0.549±0.006	18.26±0.87	1.22±0.013
F9	31.48±0.52	0.418±0.002	0.517±0.003	19.22±0.76	1.24±0.012

Data are expressed as mean ±SD (n=3)

4.5.2.5. Quality Evaluation of Tablets

After preparation of the tablets by direct compression method all the formulation batches (F1- F9) were evaluated for quality control parameters. All the parameters showed good results for all the 9 formulation batches. (Table 36)

Table 36: Quality evaluation of Herbal anti-diabetic tablets

Formulation Batch	Weight variation (mg)	Thickness (mm)	Hardness ($kg/cm^{2)}$	Friability (%)	Disintegration time (Min)	Moisture content (%)
F1	647.8±6.5	6.6±0.03	3.3±0.26	0.454±0.015	17.06±0.47	3.483±0.307
F2	649.1±6.3	6.6±0.03	6.6±0.23	0.144±0.009	25.06±0.50	3.302±0.067
F3	650.1±4.0	6.6±0.03	4.5±0.06	0.282±0.033	15.07±0.44	1.973±1.713
F4	647.1±5.4	6.6±0.03	7.3±0.29	0.133±0.009	23.02±0.46	2.617±0.253
F5	651.6±5.0	6.6±0.09	4.3±0.16	0.338±0.031	21.09±0.53	3.026±0.089
F6	648.9±6.6	6.6±0.06	7.8±0.23	0.164±0.008	24.03±0.40	3.398±0.281
F7	650.0±3.7	6.6±0.03	5.2±0.15	0.179±0.010	24.58±0.51	2.228±0.073
F8	646.3±6.1	6.6±0.01	5.9±0.15	0.261±0.016	19.20±0.26	2.619±0.055
F9	647.6±5.5	6.6±0.08	5.5±0.06	0.246±0.031	24.28±0.27	2.548±0.078

Data are expressed as mean ±SD (n=3)

4.5.2.6. Design of Experiments

A 3 level factorial design was used for the optimization and understanding the relationship between independent and dependent variables. The experimental run with independent variables and response variables obtained for the herbal anti-diabetic tablets are described in Table 37.

Table 37: Experimental design for Herbal anti-diabetic tablet formulation

Run	Conc. Of MCC	Conc. Of CCS	Disintegration time	Hardness	Friability
	X1	X2	Y1	Y2	Y3
1	50	10	17.06	3.3	0.454
2	100	10	25.06	6.6	0.144
3	50	30	15.07	4.5	0.282
4	100	30	23.02	7.3	0.133
5	50	20	21.09	4.3	0.338
6	100	20	24.06	7.8	0.164
7	75	10	24.58	5.2	0.179
8	75	30	19.20	5.9	0.261
9	75	20	24.28	5.5	0.246

4.5.2.7. Statistical optimization

The relations between independent variables and responses were analyzed using ANOVA. The results were considered to be significant at $p < 0.05$ (Table 38). Quadratic models in the form of polynomial equation were derived to ascertain the influence of the factors on the responses. Table 39 shows the polynomial equation for the measured responses. The values of the coefficients X1 and X2, interaction and quadratic-terms were related to the effect of these variables on the responses. A positive sign of coefficient indicates a synergistic effect while a negative term indicates an antagonistic effect upon the response. The larger coefficient means that the independent variable has a more potent influence on the response. To demonstrate graphically the influence of each factor on responses, the Contour plots and response surface plots are generated. (Fig 31-33)

Table 38: Statistical data for the experimental responses

Response	Sum of square	Mean Square	Degree of Freedom	F-value	P-value	Model significance
Y1	70.58	35.29	2	6.72	0.0294	Significant
Y2	16.49	8.24	2	71.34	< 0.0001	Significant
Y3	0.0685	0.0342	2	10.74	0.0104	Significant

Table 39: Polynomial equations for measured responses

Response	Polynomial Equations
Disintegration time (Y1)	**+21.40 + 3.02 *X1* - 1.63 *X2***
Hardness (Y2)	**+5.60 + 1.60 *X1* - 0.433 *X2***
Friability (Y3)	**+0.244 – 0.00422 *X1* - 0.00168 *X2***

Note: X1and X2 are independent variables where, X1– Conc. of MCC; X2– Conc. of CCS

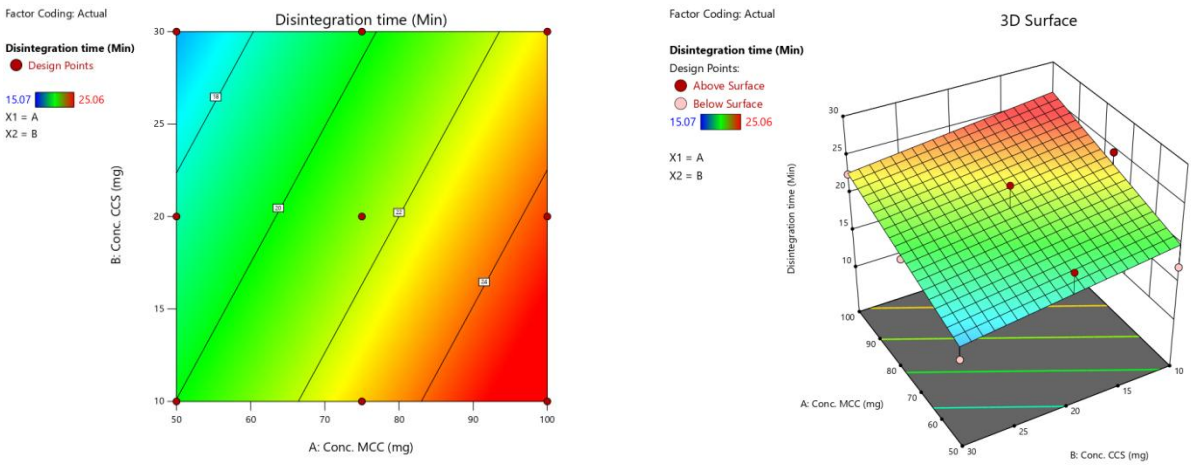


Figure 31: Contour and Response Surface Plot for response - Disintegration time (Y1)

Interpretation: the contour plot demonstrates the colored regions from blue to red indicating the regions of lower values to higher values for response Y1-disintegration time. From the 3-D response surface plot generated for response Y1 disintegration time,

an increase in the concentration of MCC resulted in the increase in tablet disintegration time whereas increasing the concentration of CCS resulted in lesser disintegration time. Thus, it can be said that both factor X1 and X2 has significant impact on response Y1.

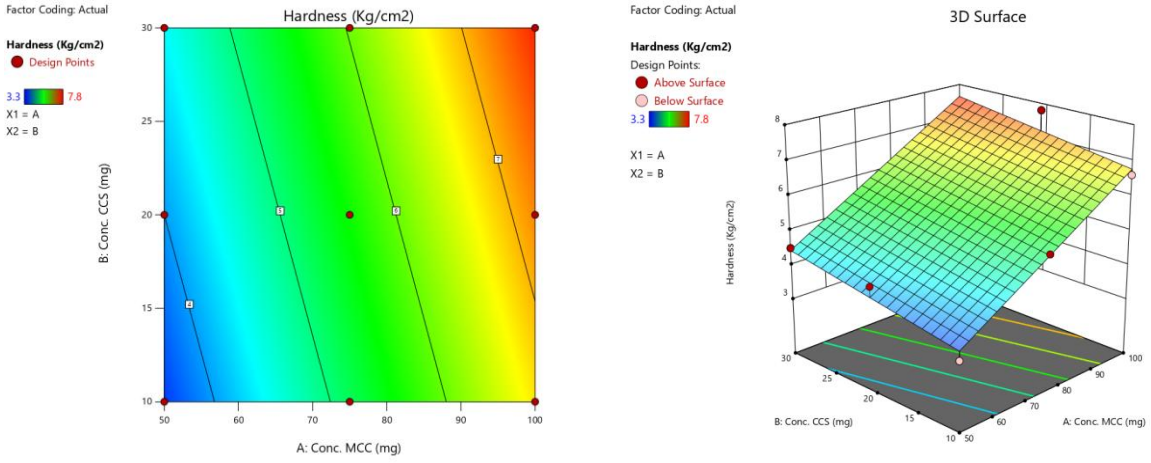


Figure 32: Contour and Response Surface Plot for response - Hardness (Y2)

Interpretation: Similarly, for response Y2 hardness, contour plot indicates the tablet hardness value ranging from lower end i.e. blue colored region to higher end marker by red color. 3-D response surface plot for Response Y2 Hardness, demonstrates an increased value for tablet crushing strength with an increase in variable X1 i.e. concentration of MCC and vice versa. Factor X2 does not seem to have a significant impact on hardness of the tablets. Hence it can be concluded that factor X1 has significant impact on response Y2.

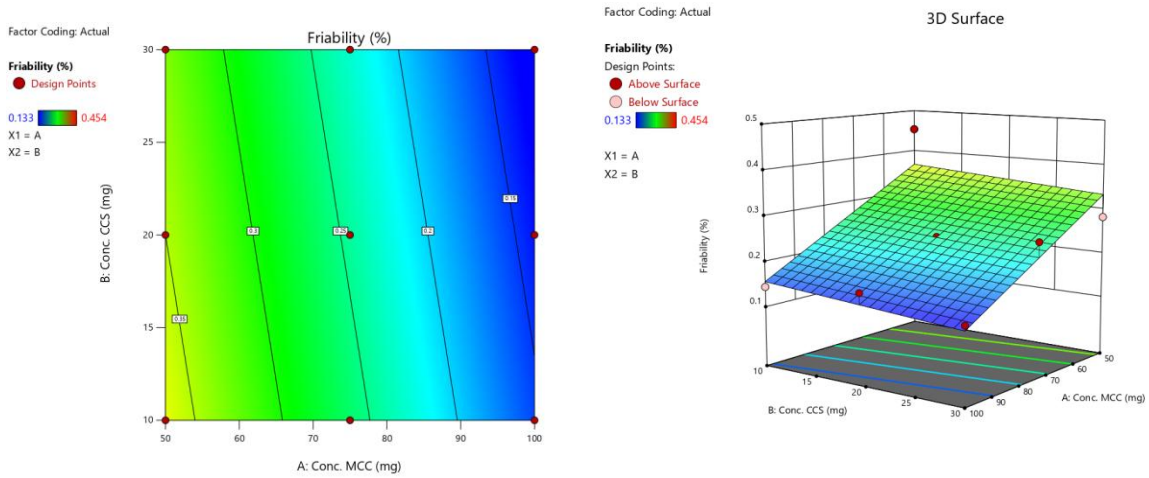


Figure 33: Contour and Response Surface Plot for response – Friability (Y3)

Interpretation: A colored region in contour plot ranging from green to blue indicates higher to lower value for response Y3 i.e. friability. From the response surface plot generated for response Y3 friability, it can be seen that increasing X1 i.e. concentration of MCC results in decreased friability of the tablets and vice versa. Hence it can be concluded that factor X1 has significant impact on response Y3.

4.5.2.8. Establishment of Design Space

The design space for herbal anti-diabetic tablet formulation was established targeting Disintegration time (Y1), Hardness (Y2), and friability (Y3). Figure 34 showed the proposed design space, comprised of the overlap region of the ranges for the three CQAs.

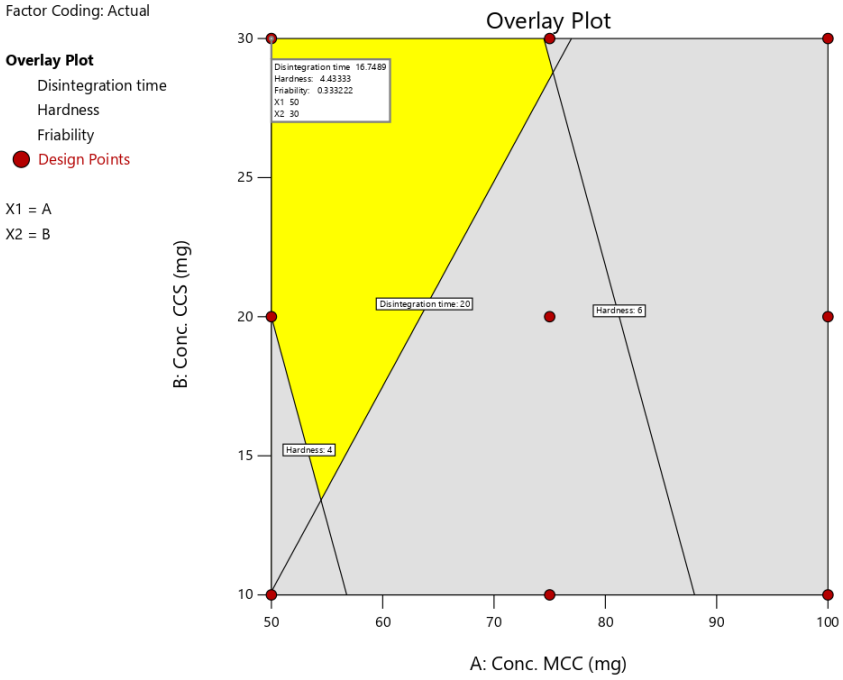


Figure 34: Overlay plot showing the Design space

The yellow shaded region is design space. This region represents the combination of factors which gives the desired tablet quality. From the design space formulation batch F3 and F5 falls under the region of successful operating ranges. Hence formulation F3 (MCC-50mg and CCS-30mg) and F5 (MCC-50mg and CCS-20mg) fulfills the criteria of QTPP and CQA for Polyherbal tablets formulation. Amongst the two formulations F3 has shown a lesser disintegration time and friability along with desired tablet hardness, therefore F3 is considered as Optimized formulation (OF).

Figure 35: Prepared tablet batches F1-F9

Figure 36: Optimized tablet formulation- F3

The optimized formulation batch F3 was selected for, further studies such as *in-vitro* drug release, short term stability studies, *in-vitro and in-vivo* anti-diabetic activity.

4.5.2.9. *In-vitro* drug release

The *in-vitro* release profile of optimized formulation batch F3 was determined successfully. Optimized Formulation batch F3 showed a good release profile with the maximum % release of GYM, TRG, CUR, DMC, BDC, and PRN were found to be 83.89, 90.72, 50.94, 48.64, 46.47, and 75.36%, respectively. The release profile of the phytoconstituents from the formulation batch F3 is represented in table 40 and figure 37.

Table 40: *In-vitro* drug release for optimized formulation F3

Time	% Drug release					
	GYM	TRG	CUR	DMC	BDC	PRN
0	0	0	0	0	0	0
15	9.0±0.19	10.19±0.96	1.39±0.02	1.97±0.05	1.30±0.02	8.90±0.19
30	19.35±1.47	20.36±0.89	3.00±0.10	3.82±0.29	3.15±0.03	20.03±0.24
60	39.48±0.84	43.85±0.55	7.28±0.03	8.47±1.23	8.96±0.26	38.88±0.21
90	60.28±1.09	66.55±2.11	13.49±0.87	16.10±0.84	15.92±0.64	59.63±0.37
120	71.44±1.08	78.30±2.60	30.28±1.14	24.70±0.42	26.99±0.25	68.62±0.91
150	80.88±0.78	86.35±2.09	43.85±0.58	36.16±0.35	34.12±0.27	72.51±1.17
180	83.89±0.82	90.72±1.25	50.94±0.40	48.69±0.52	46.47±0.56	75.36±0.85

Data is expressed as Mean±SD (n=3)

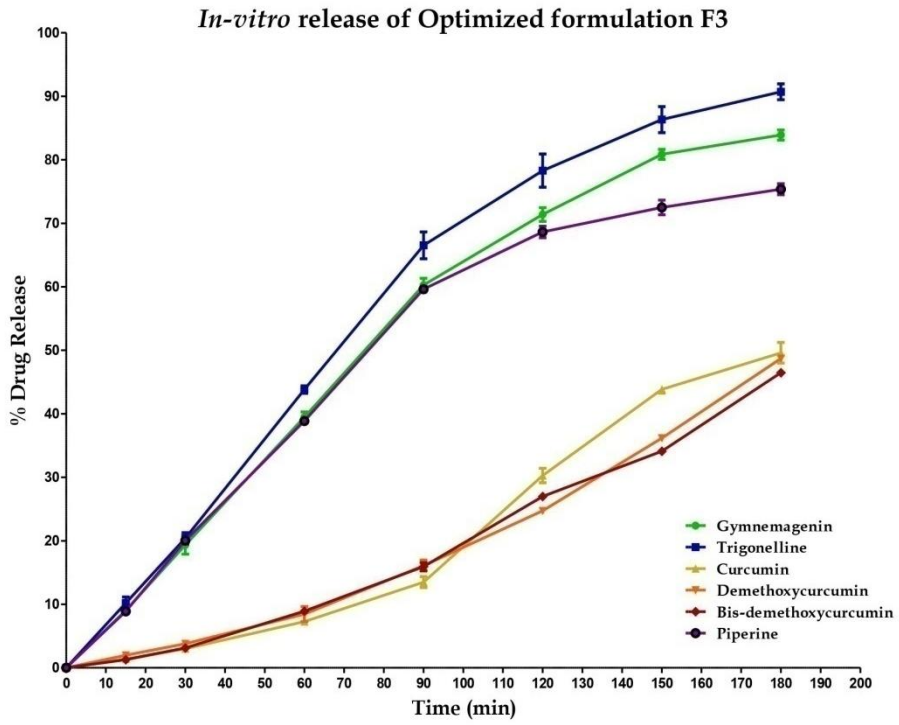


Figure 37: *In-vitro* release profile of Optimized formulation batch F3

4.5.2.10. Drug content

The assay of each marker compound in the optimized tablet formulation was determined by HPLC analysis. Based on the peak area obtained for each marker compound the % assay was calculated. Table 41 represents the % content of the marker compounds in the optimized formulation.

Table 41: Assay of optimized formulation

Marker compound	% content
Gymnemagenin	99.5±1.1
Trigonelline	101.5±1
Curcumin	102±1.1
Demethoxycurcumin	102.9±0.9
Bisdemethoxycurcumin	101.8±1.2
Piperine	99.3±1.3

Data is expressed as Mean±SD (n=3)

4.5.2.11. Stability study

The optimized tablet formulation (F3) was subjected for stability studies according to ICH Guidelines at two different temperature and RH storage conditions for three months. The tablets were analyzed for critical quality parameters at 3 time intervals during the storage period. The color and appearance of the tablets remained unchanged at both the storage conditions. Table 42 summarizes the results of stability studies of optimized anti-diabetic tablets.

Table 42: Stability study data for optimized formulation F3

Evaluation parameters	Optimized formulation F3						
	Initial	1 month		2 month		3 month	
		25 ± 2°C/60± 5% RH	40 ± 2ºC/75± 5% RH	25 ± 2°C/60± 5% RH	40 ± 2ºC/75± 5% RH	25 ± 2°C/60± 5% RH	40 ± 2ºC/75± 5% RH
Disintegration time (Min)	16.06±0.4	16.25±0.13	16.54±430	16.48±0.11	17.15±0.16	17.30±0.26	17.58±0.39
Hardness (Kg/cm^2)	4.5±0.12	4.5±0.03	4.6±0.01	4.6±0.06	5.0±0.06	5.0±0.03	5.0±0.06
Friability (%)	0.251±0.03	0.247±0.003	0.227±0.01	0.235±0.01	0.191±0.004	0.195±0.01	0.176±0.01

Data is expressed as Mean±SD (n=3)

4.6. *In-vitro* anti-diabetic assay

In-vitro anti-diabetic activity of optimized formulation F3 was determined by performing α-amylase and α-glucosidase enzyme inhibition assays. The % inhibition of each log concentration for both the enzymes is presented in Figure 38.

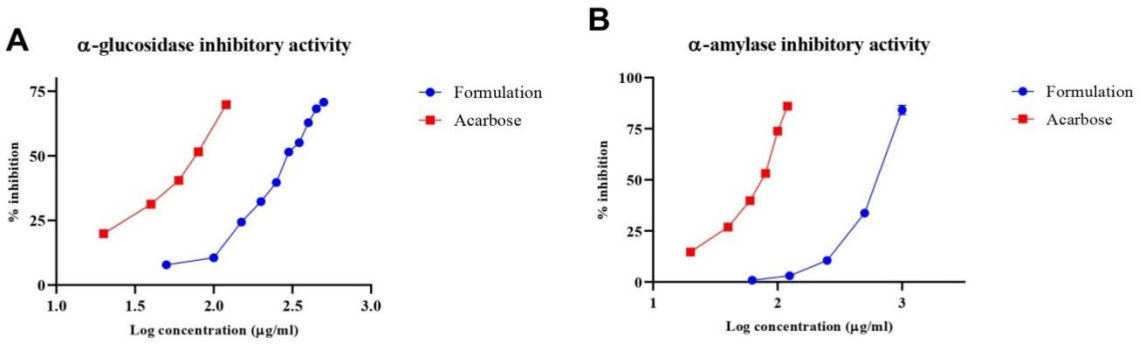


Figure 38: Percentage inhibition of α-glucosidase enzyme (A) and α-amylase enzyme (B) by F3

4.7. *In-vivo* anti-diabetic activity

4.7.1. Effect on body weight, food intake, and water intake

After the induction of diabetes, the change in body weight, food intake and water intake was observed among all the groups. Figure 39, 40 and 41 demonstrates the effect on body weight, food intake and water intake after the treatment period.

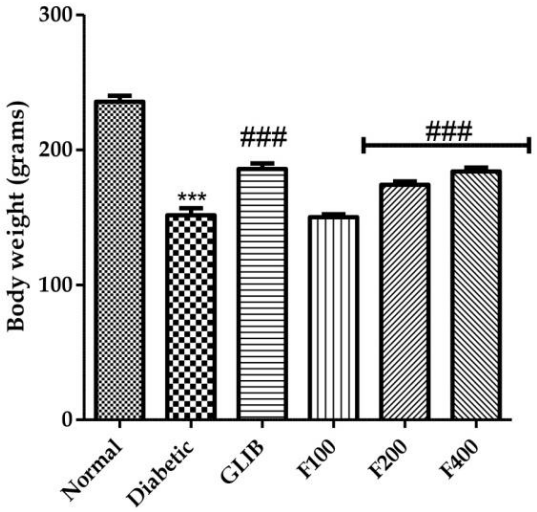


Figure 39: Effect of formulation on body weight, ***p<0.001 compared to normal, ###p<0.001 compared to diabetic group

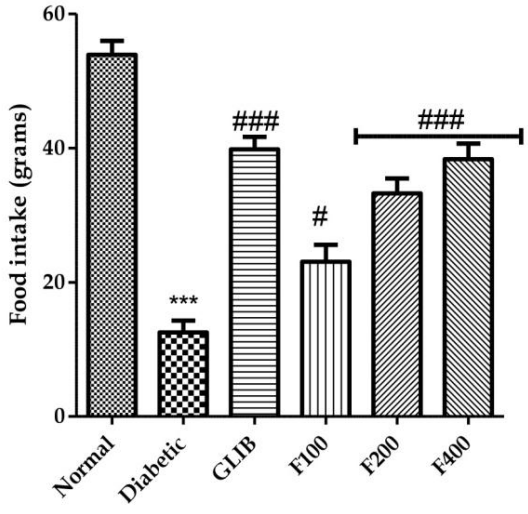


Figure 40: Effect of formulation on food intake. ***p<0.001 compared to normal, #p<0.05, ##p<0.01, ###p<0.001 compared to % change in food intake

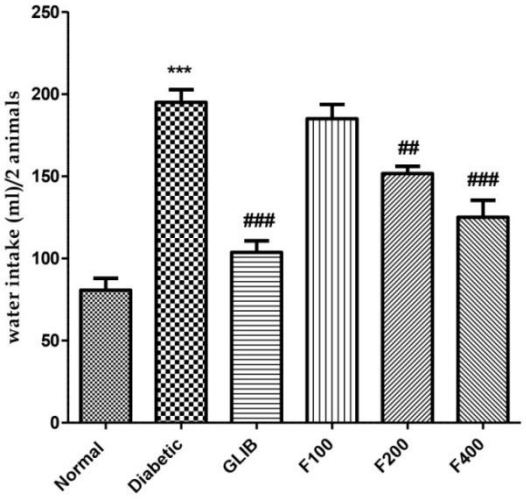


**Figure 41: Effect of formulation on water intake ***p<0.001 compared to normal, ##p<0.01, ###p<0.001 compared to Diabetic

4.7.2. Effect on Fasting blood glucose level and OGTT glucose level

After the treatment period, effect of the formulation on fasting blood glucose level and exogenous glucose clearance by performing OGTT was observed. Figure 42, 43 and 44 demonstrates the effect on fasting blood glucose level, AUC during OGTT and Effect on total AUC $_{0\text{-}120\ min}$ of glucose on OGTT respectively..

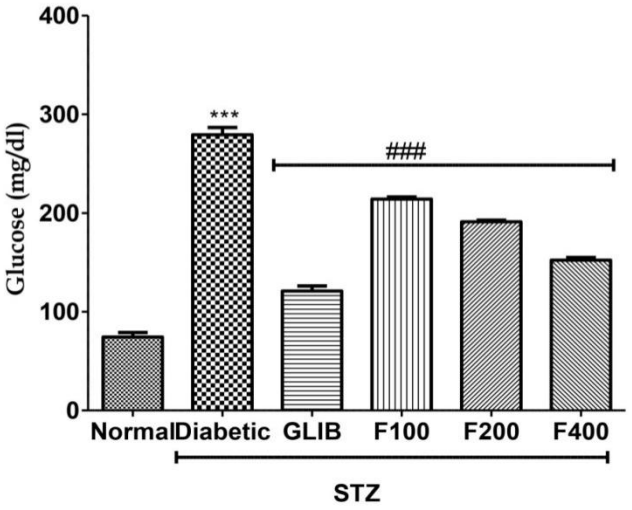


Figure 42: Effect on fasting blood glucose level. All the data are presented in mean±SEM (n=6) ***p<0.001 compared to normal, ###p<0.001 compared to Diabetic

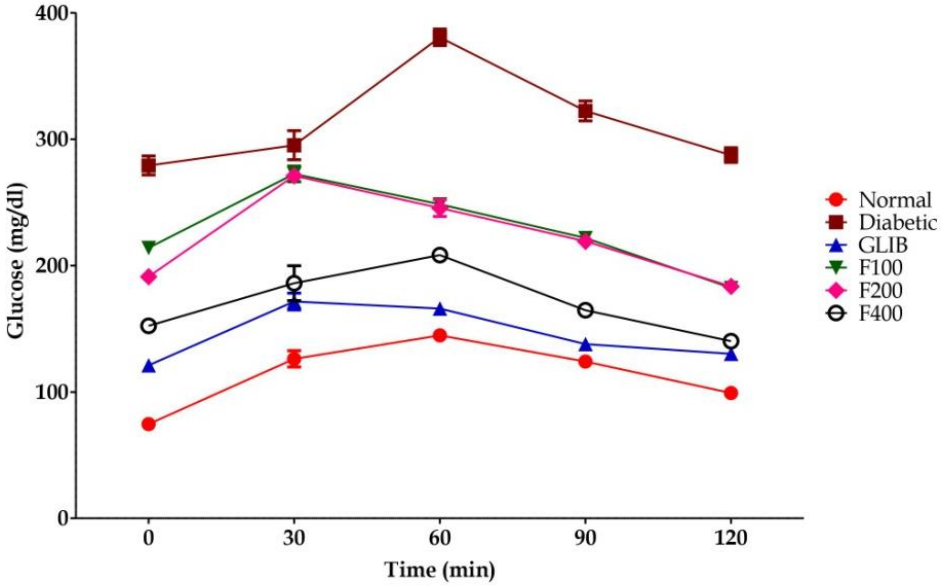


Figure 43: Total area under the curve of glucose during OGTT

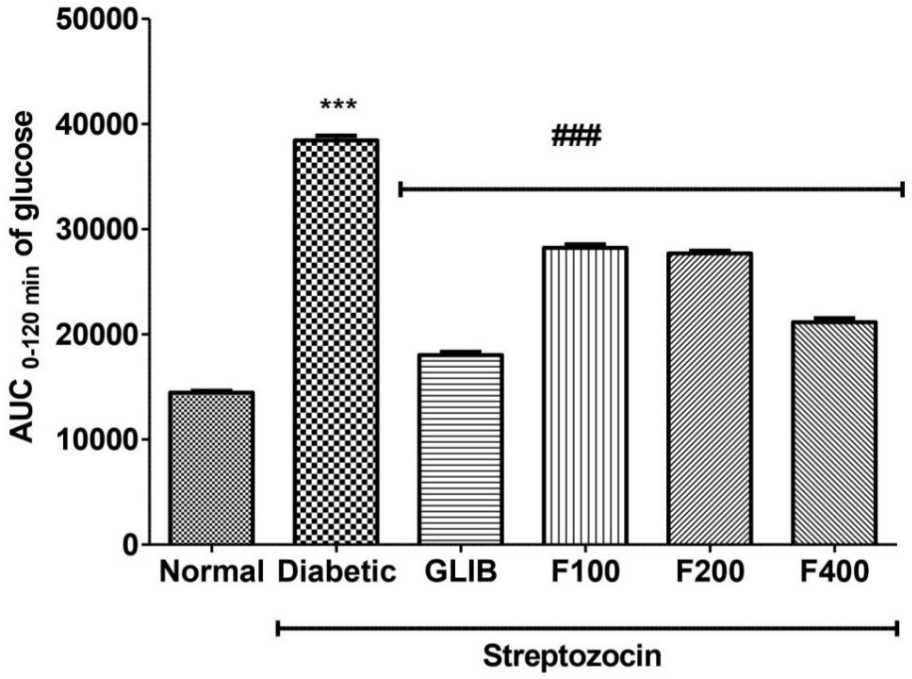


Figure 44: Effect on total AUC $_{0\text{-}120\ min}$ of glucose on OGTT

4.7.3. Effect on Lipid profile

All the groups were subjected for estimation of various biochemical parameters.

Table 43 summarizes the values obtained for estimated biochemical parameters.

Table 43: Effect of formulation F3 on lipid profile

Lipid profile	Normal	Diabetic	GLIB	F100	F200	F400
TG (mg/dL)	45.67±2.22	109.00±3.79*	49.33±2.20##	94.33±1.38##	87.00±2.35##	54.17±1.60##
TC (mg/dL)	93.50±1.41	148.70±1.99*	103.70±0.88##	121.70±1.31##	112.00±0.86##	100.50±1.86##
HDL (mg/dL)	51.33±2.53	34.50±1.52*	55.67±1.17##	42.670.95#	43.67±0.67##	52.67±1.54##
VLDL (mg/dL)	9.13±0.44	21.80±0.76*	9.87±0.44##	18.87±0.28##	17.40±0.47##	10.83±0.32##
LDL (mg/dL)	33.03±2.44	92.37±1.92*	38.13±1.45##	59.13±1.47##	41.93±1.72##	47.00±2.60##

All data are expressed in mean±SEM (n=6). *$p<0.001$ compared to normal, $^{\#}p<0.01$, $^{\#\#}p<0.001$ compared to diabetic

4.7.4. Histopathology of liver and pancreas

The histopathological examinations of pancreas are represented in figure 45.

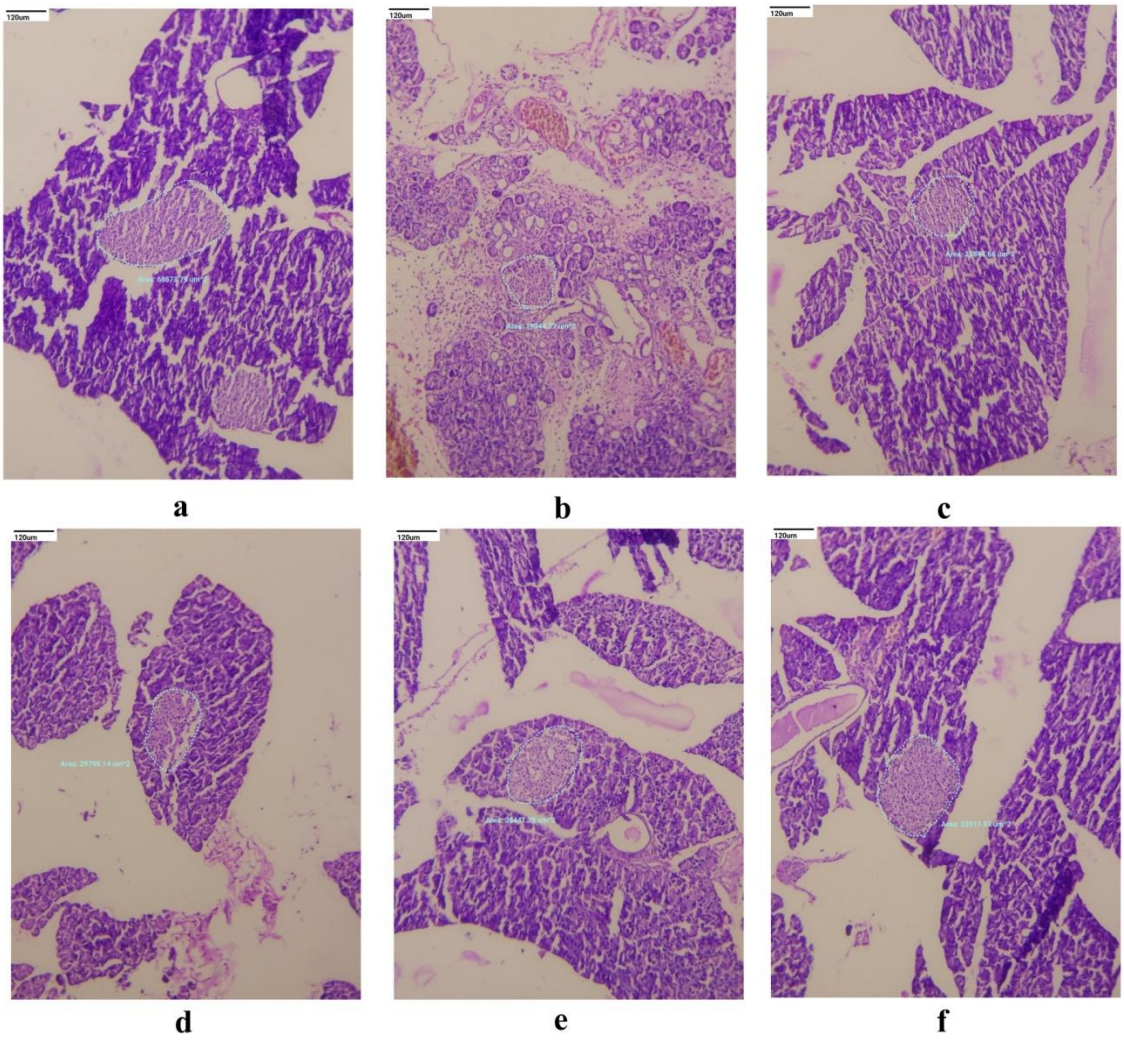


Figure 45: Effect on the pancreatic histology (a) normal, (b) diabetic, (c) GLIB, (d) F100, (e) F200, and (f) F400

5. DISCUSSION

The present study exemplifies the overall formulation development process of Herbal drugs. Currently, an increasing trend has been observed amongst pharmaceutical companies in adopting the QbD concept for manufacturing quality pharmaceutical products. The QbD is considered as a quality model in pharmaceutical manufacturing which enables the incorporation of quality at each and every step of the manufacturing process and ultimately yields products with zero defects. As we all know, natural sources of medicines have been in practice since ancient times. In the current era utilization of herbal medicines is tremendously encouraged over allopathic medicines owing to their frequent side effects. In such a scenario, stringent quality control measures should be applied for the development of herbal medicines. Hence, the present study attempts to apply the QbD approach for the development of herbal tablet formulation for treating type II diabetes.

The study is divided into three major parts involving herbal drug development. The first step is the selection and quality assessment of herbal raw material, after quality assessment of raw material second step deals with the extraction of raw material and quality assessment of the extracts, and the final step involves the development of herbal drug product i.e. tablet formulation and its quality evaluation and testing of potency.

5.1. Selection of Herbal Raw Materials

The initial step i.e. selection of the herbal raw materials were carried out as per the defined criteria. Based on the evidences from the previous literature well-known herbs reported for their anti-diabetic activity were identified. Around fifty herbs were

shortlisted by taking into consideration the criteria defined for the selection of raw material. Further, these 50 herbs were thoroughly screened mainly targeting key areas including a) quality and authenticity, b) efficacy, c) mechanism of action and synergistic effect, and d) availability of herbs, its marker compound, and cost. Finally, three herbs having potent anti-diabetic activity namely *Gymnema sylvestre, Trigonella foenum-graecum, Curcuma longa,* and an additional herb *Piper nigrum* acting as a bio-enhancer was selected for the development of anti-diabetic formulation.

5.2. Quality Assessment of Herbal raw materials

The quality of the selected herbal raw materials were assessed by evaluating botanical, Physico-chemical, chemical, and microbiological parameters. Examination of Macroscopic and microscopic characteristics is the first step towards establishing the identity of herbal crude drugs. Microscopic inspection of medicinal plant materials is indispensable for the identification of broken or powdered materials.[37] The selected plant parts were botanically identified by performing macroscopic and microscopic analyses. The morphological characters such as shape, size, color, odor, and taste of each raw material were examined. Microscopic characteristics were analyzed by performing powder microscopy.

The powdered raw sample of *Gymnema sylvestre* leaves showed the presence of a group of epidermal cells, multi-cellular trichomes, stomata, and a group of xylem vessels. Seed powder of Trigonella *foenum-graecum* revealed the presence of Cuticle, the epidermis, hypodermis of the testa, the epidermis of the testa, and parenchyma cells. Microscopic characters such as Cork cells, thickened vessel, Epidermis, and parenchymatous cells filled with yellow coloring matter were observed in *Curcuma longa*

rhizomes. Similarly, sclereids, Fibers, endocarp cells, and, a group of vessels were observed from powdered fruits of *Piper nigrum.* The observed macroscopic and microscopic characters of each raw material resemble similarly to the previously published literature.[93]

Evaluation of physicochemical properties serves as a tool for quality control and identification of crude drugs.[37] The Physico-chemical parameters such as moisture content, extractive values, Total ash value, acid insoluble ash value, and water-soluble ash value was evaluated for each herbal raw material. An excess of moisture in medicinal plant materials will encourage microbial growth and deterioration following hydrolysis. Therefore, determination of moisture content in crude drugs is an important quality control parameter. The extractive value is a vital part of the physicochemical analysis as it determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material. Similarly, Ash value is used to determine the quality and purity of the drugs as it helps in the detection of contamination or adulteration with the inorganic earthly matter.[37,38] All the physico-chemical parameters evaluated for each raw material were found to be within the specified limits.

The phytochemical analysis gives a brief idea about the presence of various secondary metabolites in medicinal plant materials. The preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, and steroids in all four raw materials. Phenols were found to be present in *G. sylvestre, T. foenum graecum, and C. longa.* Whereas saponins and glycosides were found to be present only in *G. sylvestre* and *T. foenum graecum.*

Determination of toxic substances in herbal drugs is one of the major criteria for the assessment of quality in herbal drugs. Determination of Aflatoxins, heavy metals, and pesticide residue is a prerequisite criterion for the export of herbal drugs to foreign countries. Hence this serves as a vital measure for defining the quality of herbal drug or products.[94] The Aflatoxins analysis was carried out by the HPLC method and reported the retention time for Aflatoxins standards B1, B2, G1, and G2 as 13.70, 11.35, 9.08, and 7.65 minutes respectively. The chromatogram obtained for each raw material sample does not show any significant peaks at the above-mentioned retention times, indicating the absence of Aflatoxins in the crude samples of *G. sylvestre, T. foenum graecum, C. longa* and *P. nigrum*. Further, the pesticide residues and heavy metals in all four raw material samples were found to be below the standard permissible limit. Where, the permissible limit for pesticide residues, lead, cadmium, mercury, arsenic, and chromium is not more than 0.01ppm, 10ppm, 0.3ppm, 10ppm, 5ppm, and 0.5ppm respectively.

Microbial load is an important parameter that enables the determination of microbial contamination in herbal drugs. The Microbial load in the selected herbal raw materials was determined to assess the quality and stability of the herbal raw material. The microbial load in herbal raw material was assessed in terms of total aerobic bacterial count. All the four herbal raw material samples demonstrated low bacterial count and meet the acceptance criteria specified by Indian pharmacopoeia which is NMT 10^3 CFU/gm of the dried herbal sample.

Chromatographic analysis of the selected herbal raw material was performed to quantify the phytocompounds present in them. Analytical Quality by Design (AQbD) assisted HPLC method was utilized for the quantification of phytoconstituents in each

selected herbal raw material. Phytoconstituents specific to the selected plant and reported to be responsible for the hypoglycemic effect of the herb was selected for chromatographic analysis. Amongst the various phytoconstituents present in *G. sylvestre*, gymnemic acids (GAs) are reported responsible for their anti-diabetic effects. Gymnemic acids are present in eighteen different forms (I–XVIII).[95] Hence it is very difficult to quantify all of them. Gymnemagenin is an aglycone component and a hydrolytic product of all GAs, and is commercially available. Hence, gymnemagenin was selected as a marker for the estimation of total gymnemic acid present in *G. sylvestre.* In the case of *T. foenum graecum, C. longa, and P. nigrum* Trigonelline, curcuminoids, and Piperine were selected for the quantitative estimation.

Application of the AQbD approach for the HPLC method development enabled the identification of optimized chromatographic conditions for the quantitative estimation of selected phytocompounds. The AQbD approach involved the development of the quality specification for the analytical method in terms of ATP. Based on the ATP, CQA were identified. By taking into consideration the critical analytical parameters such as the concentration of acids used in the mobile phase and mobile phase ratio, the experiments were designed by DoE tools. Here, the CQA were thoroughly examined by performing statistical analyses such as ANOVA. Further, the optimized chromatographic conditions were predicted from the MODR.

The optimized chromatographic conditions were then applied for quantitative estimation of GYM, TRG, BDC, DMC, CUR, and PRN which was found to be 8.15±0.004, 0.58±0.001, 9.32±0.20, 10.98±0.23, 13.86±0.28, and 3.63±0.06 %w/w

respectively. Further, the developed RP-HPLC methods were also validated as per ICH guidelines and all the parameters were found to be within the specified range.

5.3. Extraction

Extraction of each herbal raw material was carried out by cold maceration followed by soxhlet extraction method. Ethanol and Water were selected as solvents for the extract preparation due to their non-toxic nature. Based on the literature review the ratios of ethanol and water for each crude drug were selected in order to extract the phytocompounds of interest and to yield the maximum amount of extract. The obtained percentage yields of *G. sylvestre, T. foenum graecum, C. longa, and P. nigrum* extracts are 25.7, 26.92, 23.02, and 12.52% respectively.

5.4. Quality Assessment of herbal drug substance

Quality assessment of herbal drug substances i.e. Extracts was carried out in order to ensure the quality of the drug substance.[38] Herbal medicines are usually prepared by using extracts that act as an active pharmaceutical ingredient (API) which is ultimately believed to bring about the therapeutic effect. Subsequently, quality evaluation of these extracts is an essential part of herbal drug product development.[59] In our study, the extracts of all four herbal drugs were evaluated for moisture content, powder flow properties, phytochemical analysis, and also chromatographic analysis. The presence of a higher amount of moisture in any ingredient is considered a matter of concern in the formulation of a pharmaceutical dosage form as it can impact the stability of the dosage form.[37,38] Therefore, the moisture content in the form of percentage loss on drying was evaluated for each herbal extract. During the development of a solid dosage form, the flow property of the ingredients is considered an important parameter. Poor flow of the

formulation ingredients can hinder the tableting process. Optimum rheology of the powder (extract) is requisite for the development of a stable and quality solid dosage form.[96] Hence, the flow properties of the extracts were tested in terms of angle of repose, bulk density, and tapped density. The phytochemical and chromatographic analysis on extracts was performed to check the phytochemical nature of the extracts and loss of any phytochemical moiety during the extraction process.

The RP-HPLC method developed for the quantitative estimation of the phytocompounds in the herbal raw materials was used for the quantitative estimation of the extracts. The quantitative estimation of the extracts revealed the presence of a slightly higher amount of each phytocompounds in the respective herbal extracts. This increase in the quantity may be attributed to the used extraction solvents and extraction techniques. Moreover, previous reports have also suggested the relationship between the yield of a particular phytocompounds with the used solvent for extraction and extraction technique.[97-99]

5.5. Molecular docking

In-silico molecular docking is a significant tool in predicting the interactions between the ligand and target. Docking studies enable the prediction of bioactivity of a number of phytocompounds which paves the way for new drug development for the treatment of various diseases.[100] In our study important phytocompounds namely Gymnemagenin, Trigonelline, curcuminoids, and Piperine from *G. sylvestre*, *T. foenum graecum*, *C. longa*, and *P. nigrum* respectively were docked with the two enzymes α-Glycosidase and α-amylase which are the key enzymes in post-prandial hyperglycemia.

Among the six bioactives, Curcumin showed the highest binding affinity with α-glucosidase with the binding energy of -8.8 kcal/mol by interacting with two amino acids i.e. Thr1586 and Asp 1279 via two hydrogen bond interactions. However, the gold standard inhibitor (Acarbose) of α-glucosidase scored the highest hydrogen bond interactions i.e. 6 with Trp 1148, Thr 1150, His1449, Leu 1450, Arg 1453, Asp1454 though scored binding affinity of -8.3 kcal/mol. Similarly, among the six bioactives, Gymnemagenin scored the highest binding affinity with α-amylase with the binding energy of-8.6 kcal/mol by interacting with 4 hydrogen bond interactions with Thr163 and Glu233. Similarly, acarbose, a gold standard for the management of Post-prandial hyperglycemia scored the binding affinity of -8.2 kcal/mol; however, interacted with the highest hydrogen bond interaction i.e. 5 via His331, Asn279, Arg421, Gly334, Ser289. The order of binding affinity of the molecules with α-amylase was as Gymnemagenin>Bisdemethoxycurcumin>Curcumin>Piperine>Demethoxycurcumin>Trigonelline.

5.6. Formulation Development

5.6.1. Drug-excipient Compatibility study

Drug-excipient Compatibility study is a preliminary step in the formulation development of any pharmaceutical product. It includes the prediction of possible interaction of the drug with excipients and also defines the suitability of the excipients used in the formulation of a dosage form. The FT-IR spectroscopic analysis of the individual extract and the mixture of extract and excipients were carried out to check for the compatibility of the extracts with selected excipients. The FT-IR spectra of individual extracts and physical mixture of excipients were compared. The physical mixture

exhibited all the significant peaks that were observed in individual extract indicating no interaction of the excipients with the extracts. Hence, it can be said that excipients used in the formulation of herbal tablets are compatible with the plant extracts.

5.6.2. Quality by Design approach for Formulation development

Presently, the QbD concept is at the peak in developing quality pharmaceutical products. Almost all pharmaceutical companies are promoting the utilization of the QbD concept for formulation development. Mere testing of the products after the development process does not enhance the product quality rather it should be built into the product starting from the early product development phase as described in the QbD principles.[63]

The present study is an example of the employment of the QbD approach to the formulation development of herbal dosage forms. Thus, focuses on the consideration and application of various QbD elements for the development of herbal formulation. The first element of the QbD approach, QTPP is a summary of product quality specifications that must be achieved to ensure product quality. Therefore QTPP is designed before the initiation of the formulation process. In our study, the QTPP of herbal drug product included the details on the therapeutic indication, dosage form, dosage design, route of administration, dosage strength, important product quality attributes, container closure system, and storage condition of the product.

The second and most important element of the QbD concept is CQA. The name itself indicates the involvement of critical parameters in terms of the quality of the product. Here the attributes related to the quality of the product that is thought to be very critical so much that has the ability to impact stability and quality of the final product are accounted.[64] CQA are primarily identified from the developed QTPP. In the case of our

study, tablet disintegration time, hardness, and friability were considered as CQA of the anti-diabetic herbal tablet formulation. The developed QTPP of our herbal dosage form affirmed the development of conventional tablet dosage form and hence, disintegration time, hardness, and friability of the tablets were thought to be an important pharmacopoeial specification that needs to achieve within a given limit to ensure product quality.

After the successful development of QTPP and CQA, the next step is to conduct risk assessment and analysis. Risk assessment is done to measure the impact of each parameter involved in the product development process.[67] The initial risk assessment of herbal tablet formulation was conducted by using an Ishikawa (Fishbone) diagram and a relative risk-ranking system. The Ishikawa (fishbone) diagram was constructed by taking into consideration the important parameter involved in the formulation of herbal anti-diabetic tablets. Factors such as API in the case of our study, the extracts attributes, material attributes (excipients), process parameters, product performance, equipments used, and manufacturing environment were included in the Ishikawa diagram. These factors were further subdivided into parameters that are known to affect the final product quality directly or indirectly. All the parameters included in the diagram were studied thoroughly with respect to final product quality. The candidate factors which are predicted to be having a greater impact on final product quality were further evaluated using a relative risk-ranking system.

From the Ishikawa diagram, API i.e. extract attributes and material attributes were thought to be important factors affecting final product quality. Hence, these two factors were subjected to a relative risk ranking system. Here, each parameter falling under the

respective factor was related to the CQAs and relative risk on the final product quality was predicted in terms of high, medium, or low risk indicated by red, yellow, and green color code respectively. In the case of API attributes quantity, flow property, and moisture content of the extracts used were evaluated with respect to disintegration time, hardness, and friability of the tablets. Similarly, for material attributes concentration of excipients i.e. MCC, CCS, anhydrous lactose, magnesium stearate, and talc used in final formulation was assessed with respect to the CQA.

After thorough examination, the moisture content of the extract was considered as a high-risk factor in terms of CQA, as the higher moisture content in extracts can lead to sticking of the extract which can yield tablets with more hardness which will ultimately delay disintegration of the tablet. Therefore, the moisture content in the extracts was controlled during formulation. Amongst material attributes, the concentration of binder i.e. MCC and disintegrating agent CCS were labeled as higher risk factors. An increase or decrease in the concentration of binder and disintegrating agent can impact tablet hardness, disintegration time, and friability of the tablets. The optimization of these two higher risk factors was required to be necessary to ensure the quality of the tablets. Hence, these factors were further subjected to the DoE.

Design of Experiments (DoE) was used to evaluate the high-risk factors for predicting optimized conditions for the preparation of tablet dosage form. A 32 full factorial design was adopted for designing the formulation trial. The two high-risk factors, the concentration of MCC and CCS were chosen as independent variables denoted as X1 and X2 respectively. The selected CQA i.e. disintegration time, hardness, and friability were chosen to be dependent variables or response variables denoted by Y1,

Y2, and Y3 respectively. The independent variables were evaluated at three different levels such as low (-1), medium (0), and high (+1) for designing experimental trials. A total of nine experimental trials were designed by DoE and accordingly the formulation batches were prepared and evaluated for quality attributes, especially the selected CQAs.

The direct compression method was used for the preparation of Herbal anti-diabetic tablets. The powder blend prepared by mixing extracts and excipients was evaluated for flow properties prior to tablet compression. The powder blend for all formulation batches (F1 to F9) showed the angle of repose between 30.14°-31.84°. The loose bulk density and tapped density of all the formulation batches varied from 0.388-0.487g/cm3 and 0.473-0.591g/cm^3 respectively. Hausner's ratio was found to be in the range of 1.21-1.25. The compressibility index was found to be within 17.53-20.06. The pre-compression parameters showed good results indicating good flow characteristics of the powder blend for all formulation batches.

All the formulation batches were evaluated for physical appearance and color. The formulated tablets were found to be round biconvex in shape with a smooth texture, and brownish in color. The quality of the compressed tablets was evaluated by performing various pharmacopoeial parameters. The average weight of the tablets prepared was in the range of 647±6 to 651±5 mg. The thickness of the tablet was 6.6 mm. The disintegration time, hardness, and friability of the tablets were found to be within15.07-25.06 min, 3.3- 7.8kg/cm2and 0.454-0.133 % respectively. Since the moisture content was considered to be a major risk factor related to extracts, the presence of moisture in the tablets was evaluated in terms of percentage loss on drying which was found to be between the ranges of 1.97 % to 3.48 %.

From the quality evaluation parameters, the data obtained for each CQA was implemented in DoE. The values of independent variables and response variables were then evaluated statistically by performing ANOVA with the help of Design Expert software version 13.0.

The results were considered to be significant for each response variable at the $p < 0.05$ level. The influence of each independent variable on the dependent variables was assessed with the help of Contour plots and 3-D response surface plots. The contour plot generated for dependent variables Y1, Y2, and Y3 depicts the colored region from blue to red indicating the ranges of lower to higher response values obtained for each experimental trial.

Further, the 3-D response surface plot for response Y1 disintegration time indicates an increase in disintegration time with increasing concentration of MCC (X1). However, a decrease in disintegration time was seen by increasing the concentration of CCS (X2). Suggesting, a significant impact of both factor X1 and X2 on response Y1.

In the case of Response Y2 Hardness, it is evidenced from the 3-D surface plot that factor X1 has a significant impact on response Y2. Increasing the concentration of MCC (X1) leads to a higher value for tablet crushing strength and vice versa. Whereas, factor X2 does not seem to have any effect on the hardness of the tablets. Correspondingly, X1 has demonstrated a significant impact on Y3 when compared to X2.

The interaction between the independent variable on the CQA gives rise to the region called design space. Design space consists of the optimized region wherein selection of any combination of input variables will give rise to the product as per predefined quality specification or which will meet all the criteria of the QTPP and

CQA.[68] The design space for herbal anti-diabetic tablet formulation was developed by taking into account selected CQAs. The region wherein the experimental trials fulfill the specifications of all the three CQAs i.e. disintegration time (Y1), hardness (Y2), and Friability (Y3) is indicated by the yellow region is the design space for the herbal anti-diabetic tablet formulation. The design space of herbal anti-diabetic tablet formulation shows formulation batch F3 and F5 to be optimized. F3 consists of a lower level (-1) of independent variable X1 and a higher level (+1) of X2. Similarly, F5 consists of a lower level (-1) of independent variable X1 and an intermediate level (0) of X2. These two formulation batches fulfill the specifications of QTPP and CQA for herbal tablets formulation. Amongst the two formulations batches, F3 has shown a lesser disintegration time and friability along with desired tablet hardness and was selected as an optimized batch. Hence formulation batch F3 was subjected for the stability studies and *in-vitro* and *in-vivo* anti-diabetic activity.

5.6.3. *In-vitro* drug release Study and Drug content

The drug release profile was studied for the optimized batch F3. The herbal anti-diabetic tablets consist of a mixture of *G. sylvestre, T. foenum graecum, C. longa*, and *P. nigrum* extracts therefore, phytocompounds previously quantified in each herbal extract were considered for studying the release rate. The release rate of GYM, TRG, CUR, DMC, BDC, and PRN was determined for three hours. Amongst the six phytocompounds, Trigonelline showed a higher release of 90.7±1.25% from tablet formulation within three hours. The order of percentage release of the phytocompounds were observed as TRG (90.72±1.25%) >GYM (83.89±0.82)> PRN(75.36±0.85%)> CUR(50.94±0.40%)> DMC (48.64±0.52%)> BDC (46.47±0.56%). The release profile of CUR, DMC, and BDC was

found to be lesser when compared to the rest three phytocompounds. This may be due to the poor solubility of the curcuminoids in the dissolution media.

The drug content of the optimized tablet formulation was determined in order to ensure the presence of a stated amount of active ingredients in the formulation. The percentage drug content of optimized herbal tablets was performed by calculating the assay of phytocompounds present in it. The results demonstrated 99.5±1.1, 101.5±1,102±1.1, 102.9±0.9, 101.8±1.2, and 99.3±1.3% content of GYM, TRG, CUR, DMC, BDC, and PRN respectively. All the phytocompounds were observed to be present in specified quantities suggesting the content uniformity in the tablets.

5.6.4. Stability studies

Stability studies were carried out in order to determine the optimum storage conditions for preserving the drug's physical and chemical integrity. The stability study results indicated that herbal anti-diabetic tablets were stable when exposed to different temperature conditions for 3 months. No substantial changes were observed within the in-vitro disintegration time, hardness, and friability of the tablets.

5.7. *In-vitro* anti-diabetic assay

The anti-diabetic potential of the optimized tablet formulation was evaluated in-vitro by enzyme inhibition assays such as α-glucosidase and α-amylase inhibition assay. *In-vitro* α-glucosidase inhibitory activity reflected the inhibition of α-glucosidase with an inhibitory concentration (IC_{50}) at 291.70 ±4.22 µg/ml by the formulation and 70.95 ±0.69 µg/ml by acarbose. Similarly, the IC_{50} for the inhibition of α-amylase was found to be 572.00±22.01 µg/ml by the formulation and 61.32 ±0.5167 µg/ml by acarbose.

5.8. *In-vivo* animal activity

Prior to the injection of the STZ, the animals were randomized into 6 groups containing 6 animals in each. After the successful induction of diabetes by injection of STZ, there was a significant decrease ($p<0.001$) in body weight compared to the normal group. Treatment with glibenclamide (GLIB) and various formulation doses (F200 and F400) showed a significant increase ($p<0.001$) in the body weight as compared with the diabetic group.

Similarly, a significant decrease in food intake ($p<0.001$) and a significant increase in water intake were observed in the diabetic group when compared to the normal group. After the treatment with glibenclamide ($p<0.001$) and formulation (F100, F200, and F400) ($p<0.05$-0.01) a significant increase in food intake and a significant decrease in water intake was observed when compared to the diabetic.

5.8.1. Effect on Fasting blood glucose level and OGTT glucose level

A significant increase ($p<0.001$) in fasting blood glucose level in the diabetic group compared to normal was observed, which was significantly reversed after the treatment with Glibenclamide and formulation treatment at the dose of 100, 200, and 400 mg/kg ($p<0.01$).

During OGTT, the exogenous glucose clearance was observed to be greater with glibenclamide and 100 mg/kg, 200 mg/kg, and 400 mg/kg of formulation treatment. There was a significant increase ($p<0.001$) in total AUC of glucose in diabetic compared to normal was observed. Further, treatment with glibenclamide and different formulation doses the AUC was decreased when compared to the diabetic group.

5.8.2. Effect on Lipid profile

There was a significant increase ($p<0.001$) in TG, TC, VLDL, and LDL in a diabetic group which was significantly reversed ($p< 0.001$) within GLI5 and F100, F200, and F400 group. In contrast, there was a significant decrease ($p<0.001$) in HDL level within the diabetic group which was significantly reversed ($p<0.01, 0.001$) within the GLIB, F100, F200, and F400 group.

5.8.3. Histopathology of pancreas

There was a decrease in pancreatic beta-cell count and size in pancreatic tissue in the diabetic group when compared to the normal group. However, treatment with formulation (F100, F200, and F400) showed an increase in their count and size compared to the diabetic group.

6. SUMMARY

Diabetes mellitus (DM) is one of the most prevalent diseases in all parts of the world. The management of DM has become a global concern. From ancient times herbs are known as the important source of medication for the treatment and management of diabetes. An increasing trend has been observed in the consumption of herbal medicines for controlling DM. This can be mainly due to the complications and side effects associated with the current allopathic treatments used for diabetes. However, lack of quality assessment, inefficient processing techniques, poor quality control procedures, and lack of appropriate standardization of herbal drugs hinder the safety and quality of the finished herbal products.

Quality by design concept is a newer quality model adopted by pharmaceutical companies to guarantee the development of products with specified quality and zero defects. The QbD paradigm can play an important role in thorough understanding the herbal drug development process and circumventing the risk factors associated with it. In the proposed research work, QbD based development and evaluation of anti-diabetic herbal drug products was undertaken.

Based on the though literature review the herbal raw materials known to possess anti-diabetic activity were screened and a total of fifty herbs were enlisted. These fifty herbs were further thoroughly investigated with respect to their quality, authenticity, efficacy, mechanism of action availability of herbs, its marker compound, and cost. Amongst them three herbs having potent anti-diabetic activity namely *Gymnema sylvestre, Trigonella foenum-graecum, Curcuma longa,* and an additional herb *Piper nigrum* acting as a bio-enhancer was selected for the development of anti-diabetic

formulation. The selected four herbal raw materials were then subjected to quality assessment. In this, the four herbal raw materials were evaluated for macroscopic, microscopic, and Physico-chemical parameters. Chemical evaluation including phytochemical screening and determination of toxic contaminants such as aflatoxins, pesticide residue, and heavy metals was performed. Microbial load in terms of the total aerobic bacterial count was evaluated. AQbD assisted HPLC method was developed and validated for the quantification of Gymnemagenin, Trigonelline, Curcuminoids, and Piperine in the raw samples of *G. sylvestre, T. foenum-graecum, C. longa*, and *P. nigrum* respectively.

After quality assessment, each selected herbal raw material was subjected to extraction by cold maceration followed by the soxhlet extraction method using ethanol and water as extraction solvents. The so obtained extracts from each herbal raw material were evaluated for moisture content, powder flow properties, phytochemical analysis, and chromatographic analysis. Previously developed AQbD based HPLC method was used for quantitative estimation of phytocompounds in the respective extracts.

The *In-silico* molecular docking studies were performed wherein important phytocompounds from each selected plant were docked with the two enzymes α-glucosidase and α-amylase to predict their binding affinity with these enzymes.

Formulation of herbal anti-diabetic tablets was executed with the help of the QbD paradigm. Initially, QTPP was developed by taking into consideration the important quality specifications of the herbal drug product. CQAs were then identified from the developed QTPP which were disintegration time, hardness, and friability. Further, the risk assessment for the formulation of herbal tablets was conducted by using an Ishikawa

(Fishbone) diagram and a relative risk-ranking system. The high-risk factors screened out from the risk assessment program were subjected to DoE to ascertain their effects on the CQAs. A 3^2 full factorial design was utilized for designing the formulation trials, giving a total of 9 formulation batches. All 9 experimental batches were formulated and evaluated for Quality control tests specified by Indian pharmacopoeia. The values obtained for the CQAs after performing the experiments were analyzed statistically with the help of design expert software to predict the best fit model. The polynomial equations were also derived indicating the relationship between the independent and dependent variables. Contour and 3-D response surface plots were generated to graphically demonstrate the effect of each independent variable on the respective CQA. Further, the design space was established by setting the targeted ranges for each CQA. The formulation batch falling under the region of design space fulfilling all the criteria of the defined QTPP was selected as an optimized batch.

The *in-vitro* drug release profile and drug content of the optimized formulation batch (F3) were studied by measuring the release rate and quantity of the important phytocompounds in the optimized tablet formulation. Similarly, the stability study of the optimized tablets was carried out for 3 months by exposing the formulation to two different storage conditions.

The anti-diabetic potential of the optimized tablet formulation was evaluated *in-vitro* by enzyme inhibition assays and *in-vivo* animal activity. The *in-vitro* studies were performed by testing the inhibition of the α-glucosidase and α-amylase enzymes by the optimized formulation using acarbose as the standard. The *in-vivo* anti-diabetic activity was evaluated in STZ-induced diabetic Wistar rats. The study demonstrated the positive

effects of optimized formulation in lowering the blood glucose levels, triglycerides, total cholesterol, LDL, and VLDL in diabetic rats. *In-vitro* and *In-vivo* study suggests the promising effects of formulated herbal tablets in lowering blood glucose levels in type 2 Diabetes mellitus.

7. CONCLUSION

The present research work demonstrates a comprehensive QbD approach for the systematic design and development of herbal drug products using the example of the anti-diabetic herbal tablet formulation containing standardized extracts of *G. sylvestre, T. foenum graecum, C. longa,* and *P. nigrum.* The QTPP and CQAs for the proposed formulation were outlined with the execution of formulation trials as per 3^2 factorial design.

The application QbD concept for herbal tablet formulation enabled the development of design space by thorough understanding the relationship and effects of important material attributes and process parameters on the CQAs. The correlation of these effects yielded the space wherein selection of any combination of input variables will give rise to a product as per the QTPP having the ability to meet all the criteria of the selected CQAs.

The quality assessment performed on every step of herbal product development will be useful for ascertaining the quality of the herbal drug under study. Evaluation of pharmacognostic parameters proved the authenticity of the selected herbs. Analysis of toxic contaminants in the herbal sample assured the quality and safety of the herbs. Similarly, chromatographic analysis by AQbD assisted HPLC method enabled the qualitative and quantitative determination of the important phytoconstituents from the selected herbs. This overall quality assessment protocol will help in overcoming the lacunae associated with the quality and safety of herbal drugs.

Furthermore, the *in-vitro* and *in-vivo* anti-diabetic study has demonstrated the promising therapeutic effect of the developed herbal tablet formulation in the management and treatment of type 2 DM.

Moreover, the current study will assist readers in understanding the nitty-gritty of applying the QbD concept for the development of herbal formulations, as well as providing a foundation for the standardization of herbal drugs.

8. LIMITATIONS AND FUTURE SCOPE OF THE STUDY

Limitations:

In the present study the release profile of curcuminoids from the tablet formulation was observed to be less; this is mainly attributed to the poor solubility of curcuminoids in aqueous medium. Hence, solubility enhancement of curcuminoids could have been performed for better bioavailability of curcuminoids.

Future scope:

In order to explore the anti-diabetic efficacy of the developed herbal tablet formulation in human beings, further efforts should be taken to conduct clinical studies in human volunteers.

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Animal Ethical Committee approval letter

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BELAGAVI

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
Reg.No.221/Po/Re/S/2000/CPCSEA

Date: 17/03/2021

CERTIFICATE

This is to certify that the project proposal no ...08... entitled," Quality by Design approach to formulate an Anti-diabetic Herbal Drug Product" submitted by Dr./ Mr. / Ms., Vishakha Parab Gaonkar under the guidance of Dr. K.K.Hullatti has been approved/recommended by the IAEC of KLE College of Pharmacy, Belagavi, Reg.No.221/Po/Re/S/2000/CPCSEA in its meeting dated 13/03/2021, resolution No. 30 has been sanctioned .....36..... Rats/ Mice/ Rabbits/Guinea pig (animals) sex Either under this proposal for a duration of next.....months.

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.

Authorized by	Name	Signature	Date
Member Secretary:	Dr. N.A. Khatib	MEMBER-S Institutional Animal Ethical Comm. KLES's College of Pharmacy, BELGAUM - 590010	17/03/2021
Main Nominee of CPCSEA:	Dr. Vinod Kumar C.S	CPCSEA Nominee Institutional Animal Ethics Committee KLES's College of Pharmacy, BELGAUM.	17-3-2021

List of Publications

1. **Gaonkar VP, Hullatti K, Mannur V**. Standardization of Trigonella *foenum-graecum* L. Seeds: A Quality by Design Approach. **Indian Journal of Pharmaceutical Education and Research.** 2020; 54(4):1072-1079. **Impact factor:0.638, Cite score: 1.0**
2. **Parab Gaonkar V, Hullatti K.** Quality assessment and RP-HPLC method development for estimation of curcuminoids in *Curcuma longa*: A Quality by Design approach. **Journal of Liquid Chromatography & Related Technologies**. 2021;44(1-2):95-102. **Impact factor: 1.3, Cite score: 2.1.**
3. **Parab Gaonkar V, Mannur VK, Hullatti K**. Quality assessment and Analytical Quality by Design-based RP-HPLC method development for quantification of Piperine in *Piper nigrum* L. **Future Journal of Pharmaceutical Sciences**. 2022:8:16.
4. **Gaonkar VP, Hullatti K.** Indian Traditional medicinal plants as a source of potent Anti-diabetic agents: A Review. **Journal of Diabetes & Metabolic Disorders.** 2020;19(2):1895-1908. **Cite score: 2.1**

List of Presentations

1. **Vishakha Parab Gaonkar, Kirankumar Hullatti.** Analytical QbD based development and optimization of RP-HPLC method for estimation of Piperine in *Piper nigrum*. **"24th Annual convection and National conference of society of Pharmacognosy".** Organized by VJ's College of Pharmacy, Rajahmundy, Andhra Pradesh.
2. **Vishakha Parab Gaonkar, Vinodh Kumar Mannur.** "Quality by Design assisted RP-HPLC method development and validation for quantification of Gymnemagenin in *Gymnema sylvestre*. 1st International e-conference on **"Changing Waves in Healthcare Research: Focus on Post-Covid Era"**. Organized by School of Pharmaceutical Technology, Adamas University, Kolkata, West Bengal.

Original Article

Standardization of *Trigonella foenum-graecum* L. Seeds: A Quality by Design Approach

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ABSTRACT

Aim: To standardize *Trigonella foenum-graecum* L. Seeds by developing QbD based HPLC method for identification and quantification of trigonelline in *T. foenum graecum* L. seeds, along with evaluation of various quality control parameters. **Methods:** The Analytical Target Profile and Critical Quality Attributes were determined followed by optimization of HPLC method by using 2^2 factorial design for designing the experiments for selected independent factors. Method Operable Design Region was developed for finding out the optimized chromatographic conditions. Further quality control parameters such as macroscopic and microscopic characters, physicochemical and phytochemical characterization including determination of toxic elements were carried out on the herb. **Results:** By application of QbD approach the optimized mobile phase was identified as water with 0.01% Hydrochloric acid and Methanol in the ratio of 70:30, with the flow rate of 1 mL/ min and UV detection at 263 nm. The linear model was established in the range of 2-10μg/mL with R^2 value 0.998. The retention time of Trigonelline was found to be 2.877 min and the amount of Trigonelline in *T. foenum-graecum* L. Seeds was found to be 0.58%. The inter-day and intra-day precision were less than 2%, with accuracies between 96.6-110% of the true values. The quality control parameters showed the results within specified limits and the seeds showed absence of toxic elements in it. **Conclusion:** From the above finding we can conclude that the application of QbD approach for standardization of herbal drug can serve as an important tool for development of herbal drugs with desired quality.

Key words: Quality by Design, *Trigonella foenum-graecum* L., Trigonelline, Standardization, HPLC.

INTRODUCTION

Herbal drugs have been employed in the prevention and treatment of innumerable health ailments since ancient times.[1] According to an estimate of the World Health Organization (WHO), "about 80% of the world population uses herbs and other traditional medicines". They are known for their safety, efficacy, cultural acceptability and lesser side effects. This has engendered remarkable upsurge in the demand for herbal medicines and a necessity has been arisen for safeguarding the quality, safety and efficacy of herbal drugs.[2]

Quality control of herbal drugs is of paramount importance. The quality standards for herbal drugs rests on a clear scientific definition of the raw material. Depending on the type of crude drug, sensory properties, physical constants, adulterants, microbiological contamination and foreign materials, such as heavy metals, pesticide residues and aflatoxins, have to be checked to prove identity and purity. To substantiate the constant composition of herbal preparations, adoption of appropriate analytical methods and suitable concepts is of utmost importance in order to establish relevant criteria for uniformity.[3] Recently, the concept of "marker-based standardization" of herbal drugs is gaining

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impetus. Marker- based standardization is acknowledged as the widely accepted methods which is based on the principle of analyses of phytochemical markers by means of sophisticated chromatographic techniques such as HPLC, HPTLC etc.[4]

Along with the use of modern sophisticated instruments utilization and application of novel quality approaches are essential for developments of quality standardization parameters for herbal drugs. According to the recent literature the Quality by (QbD) concept can serve as novel approach for quality standardization of herbal drug. In recent times, pharmaceutical companies adopting QbD as a fundamental pharmaceutical quality model.[5]

Application of QbD approach in analytics is one of the alternatives which reduces the experimental time and cost for drug analysis. QbD approach indicates exploring the quality of analytical process during the development stage itself.[6,7]

Trigonella foenum-graceum also is known as Fenugreek or methi belongs to the Fabaceae family, the plant is cultivated in India and North African countries. The seeds of the plant have a long history of usage as potent antidiabetic agent in Ayurvedic and folklore medicine.[8] The main chemical constituents of seeds are alkaloids approximately 36%, steroidal saponins, mucilage, fibers.[9] Among alkaloid content of fenugreek seed trigonelline is major phytoconstituents which is responsible for most of the activity of the herb.[10]

In the present research work, an endeavor has been made to accomplish some standardization parameters for the quality control of *T. foenum-graecum* seeds with special emphasis on analysis of phytoconstituents by application of QbD approach. Hence, the primary objective of this research work was to develop a QbD based HPLC method for identification and quantification of trigonelline in *T. foenum-graecum* seeds, along with evaluation of various Quality control parameters such as macroscopic and microscopic characters, physicochemical and phytochemical characterization including determination of toxic elements in the herb.

MATERIALS AND METHODS

Plant material and Chemicals

T. foenum-graecum seeds were procured and authenticated from Shri B. M. Kankanwadi Ayurveda Mahavidyalaya, Belagavi-Karnataka. The seeds were cleaned thoroughly with water, shade dried, converted to fine powder with the help of a blender and stored in air tight bottles. Standard Trigonelline Hydrochloride was provided as a free gift sample by Himalaya drug company, Bengaluru India. Other chemicals and reagents used in the research work were of analytical grade.

Macroscopic and microscopic study

The macroscopic and microscopic characters of *T. foenum-graecum* seeds were studied by following standard procedure as specified in WHO guidelines.[11] Macroscopic characters of the seeds was studied based on shape, size, colour, odour, taste, surface characteristics and texture. Powder microscopy was performed on the seeds for the determination of microscopic characters. The photographs of specimens were captured by using the Trinocular microscope (Metzer) with Capture Pro software (4.6).

Physico-chemical analysis

The powdered seeds were subjected for analysis of physicochemical parameters such as Moisture content, extractive value, total ash value, acid insoluble ash value and water soluble ash value. All the physicochemical parameters were carried out according to the standard official methods described in WHO guidelines.[11]

Phytochemical analysis

The powdered seeds were subjected for preliminary phytochemical analysis. In order to assess the existence of secondary metabolites such as carbohydrates, Alkaloids, glycosides, tannins, flavonoids, phenols and, proteins.[12,13]

Analysis of toxic substances

The crude *T. foenum graecum* seeds were analyzed for the presence of toxic substances such as Aflatoxins, pesticide residues and heavy metals. Evaluation of Aflatoxins was carried out on Agilent HPLC instrument as per the standard procedure.[14] Aflatoxins B1, B2, G1 and G2 were analyzed in the powdered sample. The pesticide residue in the sample were determined by using Gas Chromatography-Mass spectra (GC-MS) Instrument. The presence of total 17 pesticide contaminants were analyzed in crude seed powder. The presence of heavy metals were analyzed by Atomic Absorption Spectroscopy by following standard method. Presence of Heavy metals namely lead, cadmium, arsenic, mercury and chromium were tested in the crude powdered sample.

Quality by Design based HPLC Method development

Analytical conditions

HPLC system (Agilent technologies 1220 Infinity II LC) used for the analysis consisted of a system controller, low pressure gradient pump, solvent delivery system, degasser, manual sample injector (injection volume: 5- 20µL) and UV-Vis detector. Reversed phase C_{18}

column (5µm, 4.6mmx 250mm, ZORBAX) was used for chromatographic analysis. Mobile phase comprised of acidic Water adjusted with Hydrochloric acid and Methanol in different ratio. For the analysis of the samples the flow rate was kept as 1 mL/min and the wavelength was set at 263nm. For the analysis 20µL sample was injected into the HPLC column.

Defining of Analytical Target Profile (ATP) and Critical Quality Attributes (CQA)

The first step in Analytical QbD approach is to define the analytical target profile (ATP). For designing the ATP, the necessary characteristics that are considered to be the indicators of method performance were determined.[15] The CQA's were defined from ATP to identify satisfactory performance of the developed method and to give reliable results.

Design of Experiments (DoE)

The optimization of analytical method was performed by employing design of experiments (DoE) using statistical software's. For performing the optimization a 2^2 full factorial design involving 2 factors and 2 levels, resulting in 4 experimental runs was employed in order to ascertain the critical parameters and to set their levels for designing the experiment. The design of the study was developed using Design Expert software version 12.0, (Stat-Ease Inc., Minneapolis, MN, USA).

Two independent variables i.e., % concentration of acid in aqueous phase (X1) and mobile phase ratio (X2) were varied at two different levels that were coded for low and high (-1 and +1 respectively). The response variables i.e. tailing factor (R1) and peak width (R2) were selected for performing the experiments. The DoE software was used to gain information on the critical values required to achieve the desired response of the selected independent variables.

Establishment of Method Operable Design Region (MODR)

Method Operable Design Region (MODR) was generated based on the regression models and an estimation of the probability for failure. Further, the prediction of the optimized mobile phase was carried out using the overlay plotting showing MODR. Within the design space, all the qualifications described in the ATP are accomplished at a specified risk level. On the basis of the criteria of selected CQA's, the optimized run was chosen.

Validation of the optimized method

The optimized RP-HPLC method was validated as per ICH Q2 (R1) guidelines.[16] The described method was extensively validated with reference to linearity, LOD, LOQ, precision and accuracy.

RESULTS AND DISCUSSION

Macroscopic and microscopic characteristics of seeds

The macroscopic study of *T. foenum graecum* seeds showed that the seeds were yellow in color, they were oblong in shape, 0.2- 0.5 cm long and 0.15-0.35 cm broad (Figure 1). Texture is smooth. The seeds have a pleasant odour and are bitter in taste. The microscopic characters of the seeds are depicted in Figure 2 which

Figure 1: Seeds of *T. foenum graecum* L.

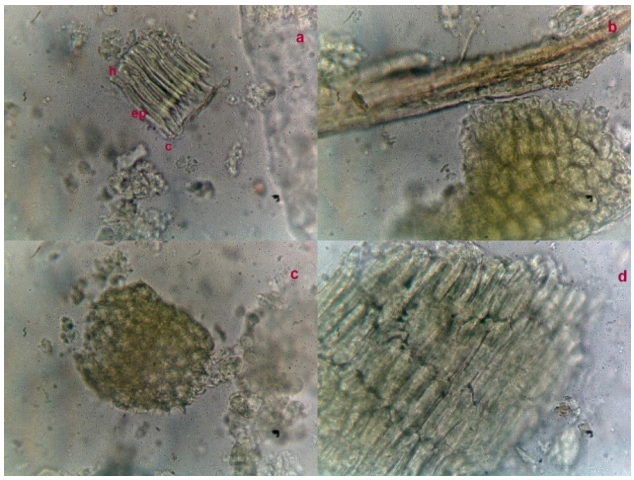

Figure 2: Microscopic characters of seed powder of *T. foenum graecum* L. showing a) Cuticle 'c', epidermis 'ep' and hypodermis 'h', of the testa, b)epidermis of the testa, c) hypodermis of the testa, d) parenchyma cells.

resembles in presence of microscopic characters as mentioned in previous literature.[17] The powdered seeds showed the presence of cuticle, epidermis, hypodermis, epidermis and parenchyma cells.

Physico-chemical analysis

The results of physico-chemical analysis of the powdered seed is shown in Table 1 along with the standard limits.[18,19] All the physicochemical parameters for crude methi seed powder showed the results within the standard limit.

Phytochemical analysis

The preliminary phytochemical analysis of *T. foenum graecum* seeds revealed the occurance of secondary metabolites such as alkaloids, flavonoids, tannins, phenols, saponins, triterpenes and steroids.

Analysis of toxic substances

The results for the analysis of toxic substances such as Aflatoxins, pesticide residues and heavy metals are depicted in Table 2.

Defining of Analytical target profile (ATP) and Critical Quality Attributes (CQA)

ATP of the proposed analytical method is to attain a good separation for quantification of Trigonelline HCl, with lesser tailing factor and peak width along with acceptable analysis time. Based on the above mentioned Analytical target profile CQA's were identified as Tailing factor (NMT 2) and Peak width (NMT 2).

Optimization of method

By performing the experiments as per the design, responses R1 and R2 were obtained for each trial and are summarized in Table 3. Further statistical optimization of analytical method was performed by comparison

Table 1: Physico-chemical parameters for *T. foenum-graecum* seeds.

Parameters	% values w/w	Standard limit[17,18]
Moisture content	7.3±0.35	NMT 9%
Aqueous soluble extractive value	33.57±1.29	NLT 30%
Alcohol soluble extractive value	21.67±0.58	NLT 5%
Petroleum ether soluble extractive value	4.40±0.53	-
Total Ash Value	3.5±0.5	NMT 4%
Acid Insoluble Ash Value	0.45±0.03	NMT 0.5%
Water Soluble Ash Value	2.83±0.29	-

NMT- Not More Than NLT-Not Less Than

Table 2: Analysis of Aflatoxins, Pesticide residues and Heavy metals of seeds of *T. foenum-graecum.*

Sr No.	Parameter	Results
1.	Determination of Aflatoxins	
	Aflatoxin B1+B2+G1+G2	BLQ (LQ: 0.2 ppb)
2.	Determination of Pesticide residue	
	DDT (Dichlora-Diphenyl-Trichloraethane)	BLQ(LOQ 0.01)
	Lindane (γ Hexachloracyclohexane)	
	α-HCH	
	β-HCH	
	δ-HCH	
	2, 4-dichlorophenoxyacetic acid	
	Endosulphon	
	Manocrotophos	
	Ethion	
	Chlorpyrifos	
	Phorate	
	Butalchlor	
	Alachlor	
	Atrazine	
	Methyl Parathion	
	Malathion	
	Aldrin	
3.	Determination of Heavy metals	
	Lead	BLQ (LOQ 1.1 mg/kg)
	Cadmium	BLQ (LOQ 0.5 mg/kg)
	Mercury	BLQ (LOQ 0.1 mg/kg)
	Arsenic	BLQ (LOQ 0.1 mg/kg)
	Chromium	BLQ (LOQ 0.5 mg/kg)

BLQ- Below Limit of Quantification LQ- Limit of Quantification

Table 3: Selected factor combinations for Trigonelline as per 2^2 full factorial design.

Code	Coded levels		Actual values		Responses	
	X_1	X_2	X_1	X_2	R_1	R_2
T1	-1	-1	0.01%	60:40	1.31	0.41
T2	-1	+1	0.01%	70:30	1.13	0.24
T3	+1	+1	0.02%	70:30	1.19	0.30
T4	+1	-1	0.02%	60:40	1.36	0.48

X_1: Conc. of HCl (%) X_2: Mobile phase ratio R_1: Tailing factor R_2: Peak width

of several statistical parameters, provided by Design-Expert® Software, Version 12. The statistical data of the applied design for Trigonelline is represented in Table 4. The independent variables and response were correlated using polynomial equations and statistical analysis through Design-Expert® Software. The coefficients X1, X2, their interaction and quadratic-terms are linked to the effect of these variables on the response.

If the coefficient is associated with a positive sign it indicates a synergistic effect whereas negative term besides the coefficient signifies an antagonistic effect upon the response. The higher coefficient indicates the potent impact of the independent variable on the response. To demonstrate graphically the influence of each independent variable on dependent variable (responses), the response surface plots were established. (Figure 3)

Establishment of the method operable design region (MODR)

The MODR for analytical method was established. MODR (Overlay plot) shown in Figure 4 had yellow color shaded region which indicates the region of successful operating ranges. From the MODR; analytical trial T2 (Conc. Of HCl 0.01% and Mobile phase ratio 70:30) and T3 (Conc. Of HCl 0.02% and Mobile phase ratio 70:30) falls under the region of successful operating ranges and fulfils the criteria of ATP and CQA for HPLC method. Among both the trials T2 having Conc. Of HCl 0.01%

Table 4: Summary of statistical parameters and polynomial equation.

Response		*P*-value	Model Significance	Polynomial equation
Trigonelline	R1	0.0272	Significant	+1.2+-0.0275*X_1-0.0875*X_2
	R	0.0268	Significant	+0.3575+0.0325*X_1-0.0875*X_2

X_1 and X_2 are independent variables where, X_1 – Conc. of Acid in aqueous phase X_2 – Mobile phase ratio

Table 5: Optimized chromatographic conditions.

Parameters	Chromatographic conditions
Stationary Phase	ZORBAX C18 (250mm x 4.6 mm, 5µ) column
Mobile phase	Water (0.01% HCl): Methanol
Mobile phase ratio	70:30
Flow rate	1ml/min
Detection wavelength	263nm
Injection volume	20µl
Retention time	2.877 min

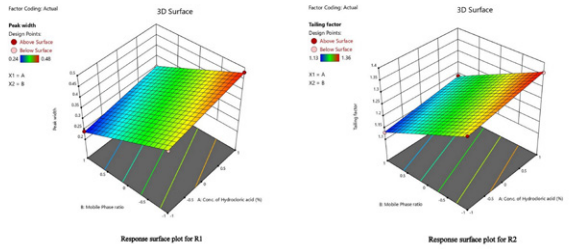

Figure 3: Response surface plot for optimization of HPLC method for Trigonelline.

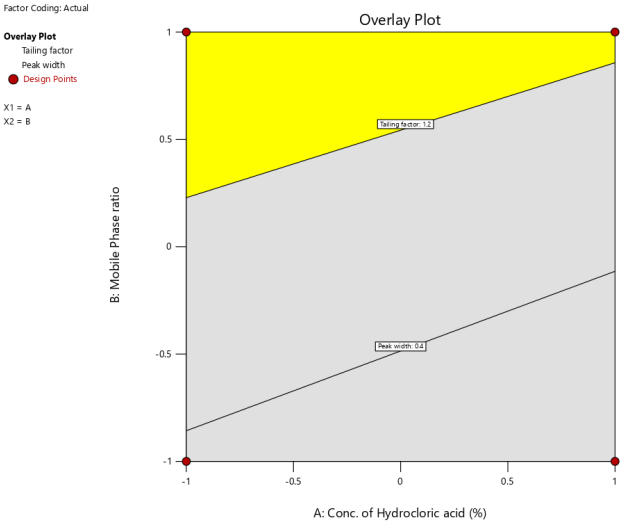

Figure 4: MODR for optimization of HPL C method for Trigonelline.

Table 6: HPLC quantification data for Trigonelline.

Marker compound	Raw material taken (mg)	Amount of marker obtained (mg)	Content of marker (%)
Trigonelline	1000	5.835	0.583

Table 7: Summary of validation parameters.

Validation Parameters		Results
Linearity range (µg/ml)		2-10
R^2		0.9981
Regression Equation		y = 307733x + 50248
LOD(µg/ml)		0.58
LOQ(µg/ml)		1.77
Precision (% RSD)	Intra-Day	1.56
	Inter-Day	1.66
Accuracy (% Recovery)		96.6-110%

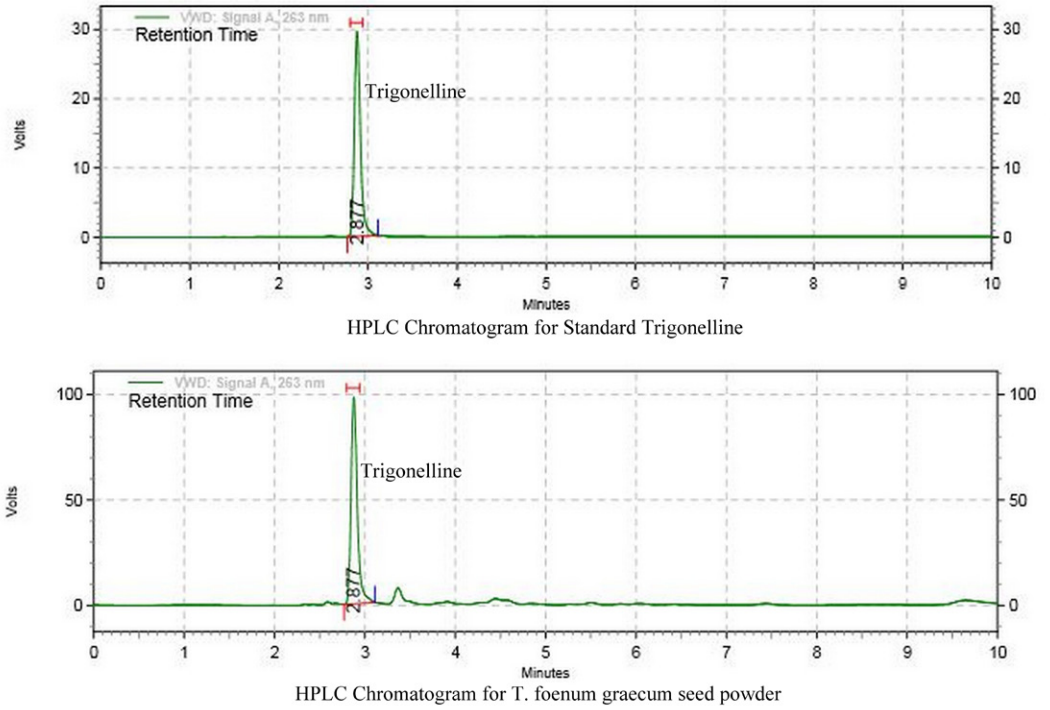

Figure 5: HPLC chromatograms of standard Trigonelline and *T. foenum graecum* L. seed.

and Mobile phase ratio 70:30 was selected as optimized HPLC method due to its ability to give lesser tailing factor and peak width Table 5.

The optimized method was further used for quantification of trigonelline in *T. foenum-graecum* seeds. The quantification data and HPLC chromatograms for trigonelline has been depicted in Table 6 and Figure 5 respectively.

Method validation

The validation of the developed RP-HPLC method was performed in order to confirm it's suitability for its intended purpose as described in ICH Q2 (R1) guidelines. The validation parameters are summarized in Table 7.

CONCLUSION

The present research work is a successful example of the implementation of QbD concept for marker-based standardization of herbal drug along with the evaluation of important quality control parameters. *T. foenum-graecum* is a valuable medicinal plant known for its usage in traditional medicine, consequently, it is imperative to standardize the herb for assessing its quality and its subsequent usage as a drug. Marker-based standardization of herbal drugs is an important technique for the assessment of the quality of herbal drugs and marked herbal products due to its ability to give an account on the phytoconstituents present in a particular plant and also to monitor batch to batch uniformity of phytoconstituents in the finished herbal products. In the present research work Trigonelline an important alkaloidal phytoconstituents has been used for standardization of *T. foenum-greacum* seeds along with the application of QbD approach. Utilization of QbD approach has assisted in developing chromatographic method which has given reproducible and reliable results along with reduction in analysis time and cost. By application of QbD approach the predefined Analytical target profile was achieved by monitoring the critical quality attributes. Further the quality control parameters for *T. foenum-greacum* seeds was accomplished as the morphological, physicochemical and phytochemical evaluation demonstrated results within the standard limits. In our study with seeds of *T. foenum-graecum*, the toxic contaminates profile was found to be satisfactory with amounts of aflatoxins, pesticide residues and heavy metals being below limit of detection.

From the above finding we can conclude that the application of QbD approach for standardization of herbal drug can serve as an important tool for development of herbal drugs with desired quality. Further the standard parameters studied in this research work will be beneficial for confirming the authenticity

of this valuable herb and also will pave a way for ensuring and maintaining the quality of crude drug.

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CONFLICT OF INTEREST

The authors declare no Conflict of Interest.

ABBREVIATIONS

QbD: Quality by Design; **HPLC:** High Performance Liquid Chromatography; **ATP:** Analytical Target Profile; **CQA:** Critical Quality Attributes; **MODR:** Method Operable Design Region; **DoE:** Design of Experiments.

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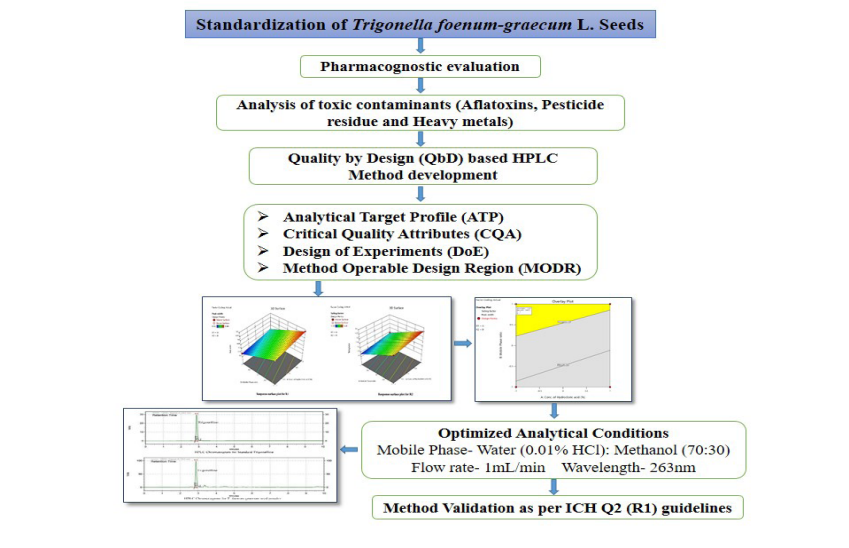
PICTORIAL ABSTRACT



SUMMARY

In the present research work standardization of *Trigonella foenum-graecum* L. Seeds was carried out by developing QbD based RP-HPLC method. Initially, the powdered seeds were subjected to Quality control evaluation and examination of toxic contaminants such as Aflatoxins, pesticide residues and heavy metals. Further, by using QbD principles ATP and CQA's were defined for the development of the RP-HPLC method. Depending on the developed Design of Experiments (DoE) analytical trials were performed and the optimized chromatographic conditions were derived from the Method Operable Design Region (MODR). Results revealed that all the Quality control parameters were within the standard limit and the toxic contaminates profile was found to be satisfactory with amounts of aflatoxins, pesticide residues and heavy metals being below the limit of detection. By application of the QbD approach, the optimized mobile phase was identified as water with 0.01% Hydrochloric acid and Methanol in the ratio of 70:30, with the flow rate of 1 mL/ min and detection wavelength 263 nm. The retention time of Trigonelline was found to be 2.877 min and the amount of Trigonelline in *T. foenum-graecum* L. Seeds was found to be 0.58%. Hence, it can be concluded that the QbD approach can serve as an important quality tool for marker-based standardization of Herbal drugs.

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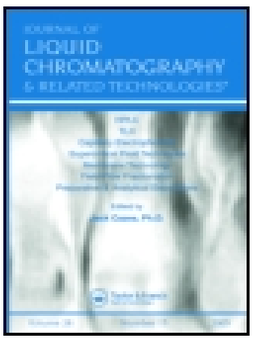

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Quality assessment and RP-HPLC method development for estimation of curcuminoids in *Curcuma longa*: A Quality by Design approach

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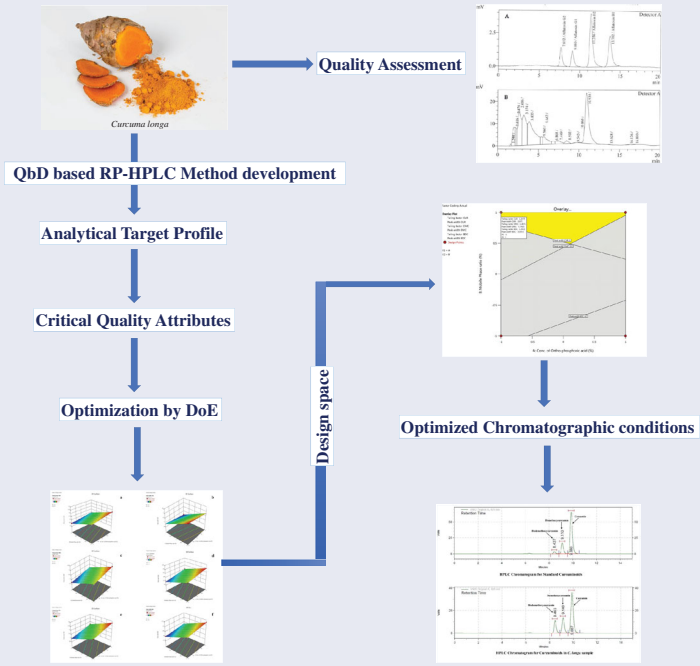
ABSTRACT

The present work aims at developing a QbD assisted RP-HPLC method for the estimation of individual curcuminoids in *Curcuma longa* rhizomes along with its quality assessment. The quality of crude *C. longa* sample was evaluated with respect to physicochemical, phytochemical and toxic contaminant analysis. The ATP and CQA's were identified followed by the optimization of the method by employing 2^2 factorial design. The concentration of orthophosphoric acid in aqueous phase (X_1) and mobile phase ratio (X_2) were selected as independent variable whereas, Tailing factor CUR (R_1), peak width CUR (R_2), Tailing factor DMC (R_3), peak width DMC (R_4), Tailing factor BDC (R_5), and peak width BDC (R_6) selected as dependent variables. The optimized chromatographic conditions were identified with mobile phase, Acetonitrile and Water (0.02%OPA) in the ratio of 55:44 with flow rate of 1 mL/min and detection wavelength of 425 nm. Further, the amount of curcumin, demethoxycurcumin and bis-demethoxycurcumin in crude *C. longa* sample was found to be 13.86 ± 0.28, 10.98 ± 0.23 and 9.32 ± 0.20 mg/g respectively. From the results obtained it can be concluded that the application of the QbD approach ensured the development of a more precise method that can engender consistent, reliable, and quality data throughout the process and also save time.

KEYWORDS

Curcuma longa; curcuminoids; quality assessment; quality by design; RP-HPLC

GRAPHICAL ABSTRACT



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Introduction

Curcuma longa has been esteemed worldwide as herbal medicine attributable to its health-promoting properties. Most of the pharmacological activities of *C. longa* are attributed to the curcuminoids. Curcuminoids comprise of a combination of three associated compounds particularly curcumin, demethoxycurcumin, and bisdemethoxycurcumin. *Curcuma longa* has been reported to possess anti-inflammatory, antibacterial, anticancer, antioxidant, antidiabetic activities. These effects are closely related to the levels of curcuminoids in the herb, hence the determination of curcuminoids is very important to assure the standardization of *C. longa* used in traditional medicine.[1,2]

Quality control of herbal drugs is a crucial task; factors such as geographic and environmental differences of growing conditions, physical constants, adulterations, and foreign materials could affect the quality and also batch-to-batch uniformity of herbal products. Consequently, quality assessment of herbal crude drugs especially concerning pharmacognostic and phytochemical parameters is essential in order to prove the purity and safety of herbal drugs.[3,4]

Numerous analytical methods have been previously reported for the determination of total and individual curcuminoids in various matrices, particularly techniques involving spectrophotometric analysis for the determination of total curcuminoids.[5]

Due to the intense yellow hue, curcuminoids are typically analyzed between 420 and 430 nm. In order to achieve accurate quantification of curcuminoids in different samples, efficient separation of components is crucial to evade mutual interferences.[6] Consequently, chromatographic techniques are deemed to be methods of choice for quantification of curcuminoids owing to their separation capacities.[7] Taking into account the ease and sensitivity of the method, High-Performance Liquid Chromatography (HPLC) techniques are widely employed for the separation of complex compounds. HPLC method is known for its high efficiency and rapid separation.[8]

Several HPLC methods for the separation of curcuminoids are reported. However, the previously reported methods are based on the time-consuming principle of varying "One Factor at a Time (OFAT)" approach. On the other hand, Quality by Design approach is gaining impetus as a newer concept for developing and analyzing quality pharmaceutical products. This approach when applied for the development of an analytical method is known as Analytical QbD (AQbD).[8,9]

The quality by Design approach is a systemic and risk-based approach supported by ICH Q8 and Q9 guidelines, which primarily rely on the principle of variation of multiple factors and identifying the risk that may lead to poor method robustness. Generation of Design space is the core concept of Analytical QbD, wherein the experimental region defined which implies that changes to method parameters will not significantly affect the results. Subsequently, this approach builds in robustness to the method as the method is being developed.[10,11]

Food and Drug Administration (FDA) has been especially dynamic in emboldening the concept of QbD which involves incorporating quality into the process and the product in a systematic, scientific, and risk-based manner during the development stage rather than endeavoring to test quality at the end after the development stage.[12]

Previously reported HPLC-UV methods for curcuminoids, have several disadvantages, including unsatisfactory separation times, poor resolution, and/or complicated solvent mixtures with gradient elution. The present work aims at developing a QbD assisted simple and rapid isocratic RP-HPLC method for the estimation of individual curcuminoids for quality control of *C. longa* rhizomes in powdered raw material form.

Materials and methods

Chemicals and reagents

Standards of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were provided as a gift sample by the Himalaya Drug Company, Bengaluru India. Acetonitrile, Ortho-phosphoric acid, and water were of HPLC grade purchased from Merck, Mumbai, India Pvt Ltd. Sample of crude *C. longa* rhizomes was procured and authenticated from Shri B. M. Kankanwadi Ayurveda Mahavidyalaya, Karnataka.

Quality assessment of C. longa rhizomes

The quality assessment of *C. longa* rhizomes was carried out by performing the quality control parameters mentioned in WHO guidelines. Parameters such as physico-chemical parameters including moisture content, extractive value, total ash value, acid insoluble ash value, and water-soluble ash value were performed along with phytochemical analysis. Further, Analysis of Toxic substances such as determination of Aflatoxins, pesticide residue, and heavy metals was carried out in the crude sample of *C. longa* rhizomes.[13–15]

Chromatographic instrumentation and conditions

The chromatographic analysis was carried out on Agilent 1220 Infinity II LC system (Agilent technologies, Germany) equipped with the system controller, low-pressure gradient pump, solvent delivery module, online degasser, manual sample injector (injection volume ranging between 5 and 20 µL), and UV-Vis detector. The Agilent OpenLab software was used for instrument control and data processing. Reversed-phase C-18 column (5 µm, 4.6mm × 250 mm, ZORBAX) was used for chromatographic separation. The mobile phase was composed of Water adjusted to acidic pH with Ortho-phosphoric acid, and Acetonitrile in different ratios. Samples were analyzed at the flow rate of 1 mL/min and the detection wavelength was set at 425 nm. For each analysis, a 20 µL sample was injected into the column.

Preparation of standard and sample solutions

A stock solution consisting of 1000 µg/mL of each Curcuminoid, i.e., Curcumin (CUR), Demethoxy curcumin (DMC), and Bis-demethoxy curcumin (BDC) was prepared using methanol. Further, different dilutions with varying concentrations (2–10 µg/mL) were prepared using the mobile

phase and filtered through a 0.22 mm membrane filter prior to their injection into the chromatographic column.

For the preparation of the sample, accurately weighed 1 g of powdered samples of *C. longa* rhizomes was exhaustively refluxed for 1 hr with 50 mL of methanol. The solution was filtered and marc was again refluxed for another 1 hr with 50 mL of methanol and filtered. Both the filtrate were combined and diluted to 100 mL with methanol and were used for further analyses.

Quality by design-based method development

Defining of analytical target profile (ATP) and critical quality attributes (CQA)

Application of the Qbd approach for the analytical method development entails the prior outlining of Analytical Target Profiles (ATP). Wherein, the essential characteristics that are considered to be the indicators of method performance are to be determined.[16] In order to accomplish reliable results, Critical Quality Attributes (CQA's) has to be identified from the defined ATP which will be useful in ascertaining the satisfactory performance of the developed method.

Optimization of method using design of experiments (DoE)

The HPLC method was optimized by employing DoE for studying the effect of selected process parameters and their responses. A simple 2^2 full factorial design with 2 factors and 2 levels, resulting in 4 experimental runs was employed in order to identify the optimized chromatographic method. The design of the experiments was developed using Design-Expert software version 12.0, (Stat-Ease Inc., Minneapolis, MN, USA).

The DoE software was used to gain information on the critical values required to achieve the desired response of the selected independent variables.

Establishment of method operable design region (MODR)

Method Operable Design Region (MODR) was generated based on the regression models and an estimation of the probability for failure to ascertain its suitability. Further, the prediction of the optimized mobile phase was carried out using the overlay plot showing MODR. Within the design region, all the specifications mentioned in the ATP are fulfilled at a specified risk level. The optimum run was selected based on the criteria of selected CQA's.

Validation of the optimized method

In order to authenticate the consistency of the optimized method, method validation was performed in compliance with ICH Q2 (R1) guidelines.[17] The optimized method was validated in terms of system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and Accuracy.

System Suitability: system suitability was assessed by injecting six replicate injection of standards (6 µg/ml), followed by estimation of % RSD for the CQA's.

Linearity: The linearity was carried out for each curcuminoids standard. Five different concentrations ranging from 2 to 10 µg/mL for each curcuminoid was injected into the HPLC system to obtain a linear dynamic range for each standard. The linearity was assessed by calculating the slope, y-intercept, and correlation coefficient (r^2) using least squares regression.

Limit of quantification (LOQ) and limit of detection (LOD): Method sensitivity was evaluated by calculating LOD and LOQ, which are dependent on the values of SD and slope of the calibration curve. LOD and LOQ were calculated as:

$$LOD = 3.3 \times \delta/S \;\; LOD = 10 \times \delta/S$$

where δ is the Standard deviation of y-intercept and S is the Mean slope of calibration curves.

Precision: The precision of the optimized method was performed in terms of intra-day and inter-day precision. Analysis of intra-day precision was done on the same day at different time intervals by determining repeatability, and on three consecutive days, by determining intermediate precision (inter-day precision) for different concentrations of analytes such as low, intermediate, and high concentrations.

Accuracy: The accuracy of the method was established by carrying out recovery studies. A known quantity of samples of raw materials was spiked in triplicate injections at low, medium, and high concentration levels of 50, 100, and 150%, respectively. The mean percentage recovery for each standard was calculated.

Result and discussion

Quality assessment of C. longa rhizomes

Assessment of the quality of herbal crude drugs plays an important role in determining safety in the usage of the crude drug. All the Physicochemical parameters displayed results within the given standard limits. The moisture content, aqueous soluble extractive value, alcohol soluble extractive value, Total ash value, acid insoluble ash value, and water-soluble ash value was found to be 6.55 ± 0.51, 13.83 ± 0.76, 12.33 ± 0.58, 6.67 ± 0.29, 1.0 ± 0.5, and 4.0 ± 0.5% w/w, respectively.

The preliminary phytochemical screening of *C. longa* rhizomes revealed the presence of important secondary metabolites such as phenols, alkaloids, flavonoids, tannins, triterpenes, and steroids whereas saponins and proteins were found to be absent. Determination of toxic substances serves as one of the major criteria for the assessment of quality in herbal drugs. The chromatogram obtained for the HPLC analysis for Aflatoxins in the given sample is depicted in Figure 1. Further the results revealed the absence of all the three toxic substances in the given sample; therein advocating the quality and safety of the *C. longa* rhizomes.

Chromatographic conditions

Preliminary chromatographic trials were conducted by thorough screening of the previous scientific reports available on chromatographic separation of curcuminoids.[3,6,7,16,18–21] most of the previously reported methods suggested the utilization of organic solvents, for example, acetonitrile, methanol, etc. along with the aqueous solvent system of buffers adjusted to acidic pH with acetic acid, Sodium dihydrogen

phosphate, disodium hydrogen phosphate, Orthophosphoric acid. Hence, with the assistance of the QbD concept, we endeavored to develop an optimum method consisting of a systemic combination of Acetonitrile as organic and water with orthophosphoric acid as an aqueous phase with suitable flow rate and wavelength for the quantification of each curcuminoids simultaneously.

QbD-assisted RP-HPLC method development

The various characteristics that are certainly indicative of the method performance and its implications are collectively summarized to be known as the analytical target profile. The selection of an analytical target is based on the method goal.

Defining ATP pertaining to quality characteristics is the first step in the Analytical QbD approach. In the present study ATP of the proposed analytical method was to achieve a good separation for quantification of curcuminoids, with lesser tailing factor and peak width along with acceptable analysis time. Based on the above mentioned Analytical target profile, Critical Quality Attributes (CQA's) were identified as Tailing factor (Not More Than 2) and Peak width (Not More Than 2). The rationale behind selecting the tailing factor and peak width as CQA's rely on the accuracy of quantitation of each marker compound. Since the accuracy of quantitation decreases with increase in tailing factor therefore in order to attain precise peak integration, tailing factor and peak width have been selected. Which is further essential to obtain accurate quantification of the three selected marker compounds.

Method optimization by DoE

As per the employed 2^2 full factorial design, two independent variables, i.e., % concentration of Orthophosphoric acid in aqueous phase (X_1) and mobile phase ratio (X_2) were varied at two different levels that were coded for low and high (−1 and +1 respectively). The response variables, i.e., tailing factor and peak width was selected for performing the experiments.

The response variables tailing factor and peak width of the experimental design arising out of four chromatographic trials are summarized in Table 1. Further statistical optimization of the analytical method was performed by comparison of several statistical parameters, provided by Design-Expert® Software, Version 12. The statistical data of the applied design is

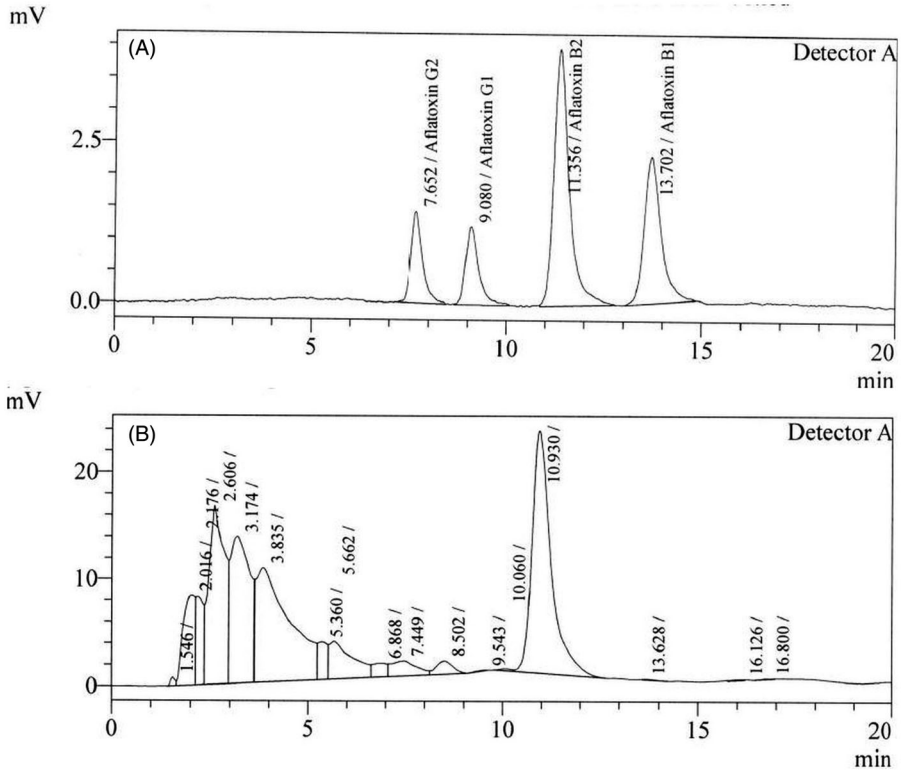

Figure 1. HPLC chromatograms for (A) standard aflatoxins and (B) *C. longa* sample.

Table 1. Experimental design for screening of independent variables and responses.

	Coded levels		Actual values		Responses					
					Tailing factor CUR	Peak width CUR	Tailing factor DMC	Peak width DMC	Tailing factor BDC	Peak width BDC
Code	X_1	X_2	X_1	X_2	R_1	R_2	R_3	R_4	R_5	R_6
Trial 1	−1	−1	0.02%	60:40	1.37	1.23	1.37	0.85	1.41	0.69
Trial 2	−1	+1	0.02%	55:45	1.25	0.97	1.27	0.74	1.29	0.6
Trial 3	+1	+1	0.05%	55:45	1.29	0.91	1.31	0.8	1.37	0.63
Trial 4	+1	−1	0.05%	60:40	1.42	1.15	1.42	0.9	1.48	0.73

X_1: % conc. of OPA in aqueous phase; X_2: mobile phase ratio

Table 2. Summary of statistical parameters and polynomial equations.

Response		*p*-Value	Model significance	Polynomial equation
Curcumin (CUR)	R_1	0.0376	Significant	$+1.33+0.0225*X_1-0.0625X_2$
	R_2	0.0385	Significant	$+1.07-0.0350*X_1-0.1250*X_2$
Demethoxy Curcumin (DMC)	R_3	0.0437	Significant	$+1.34+0.0225*X_1-0.0525*X_2$
	R_4	0.0421	Significant	$+0.8225+0.0275*X_1-0.0525*X_2$
Bis-demethoxy Curcumin (BDC)	R_5	0.0364	Significant	$+1.39+0.0375*X_1-0.0575X_2$
	R_6	0.0493	Significant	$+0.6625+0.0175*X_1-0.0475*X_2$

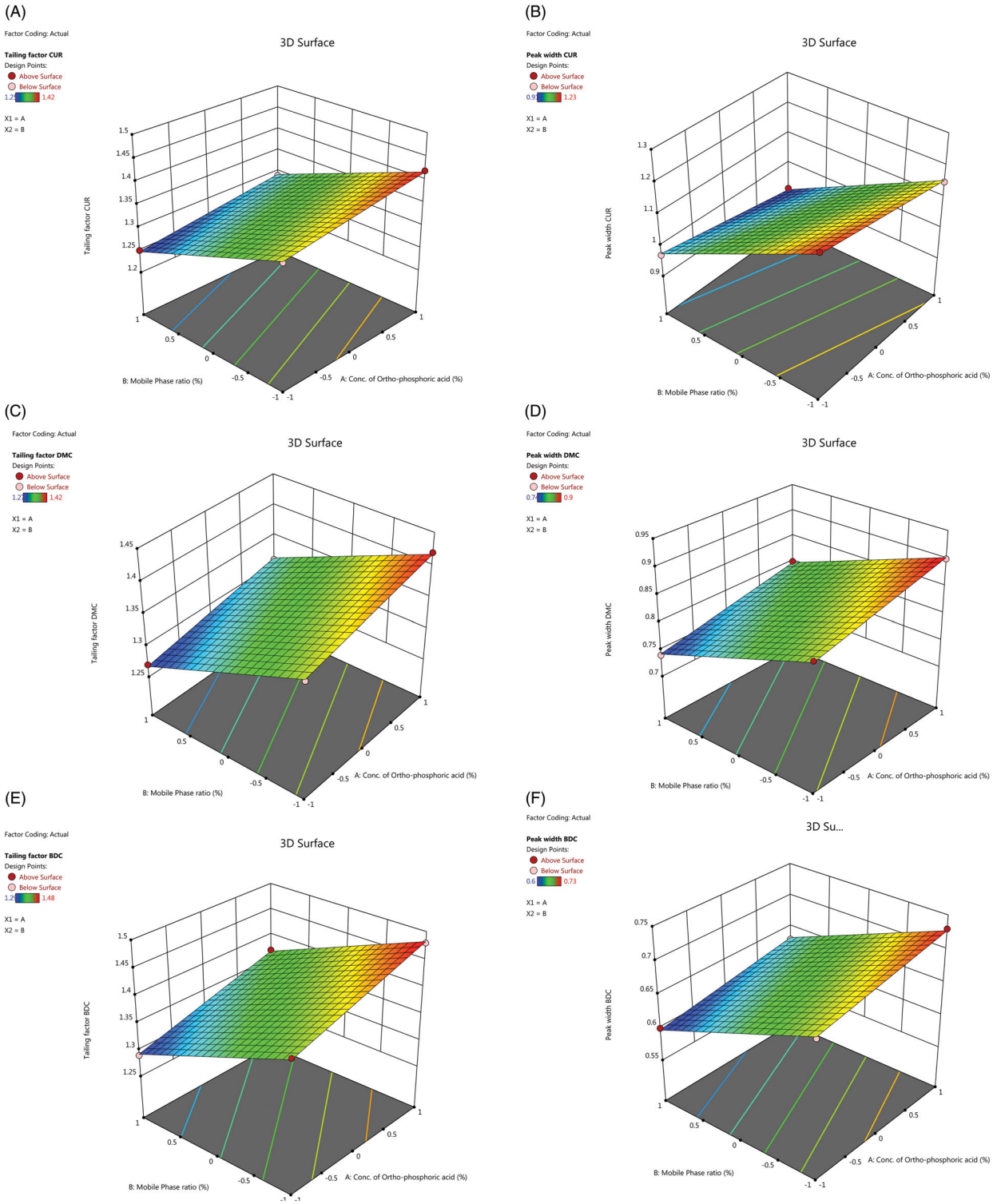

Figure 2. 3D response surface plots for dependent valuables, tailing factor CUR (a), peak width CUR (b), Tailing factor DMC (c), peak width DMC (d), tailing factor BDC (e), and peak width BDC (f).

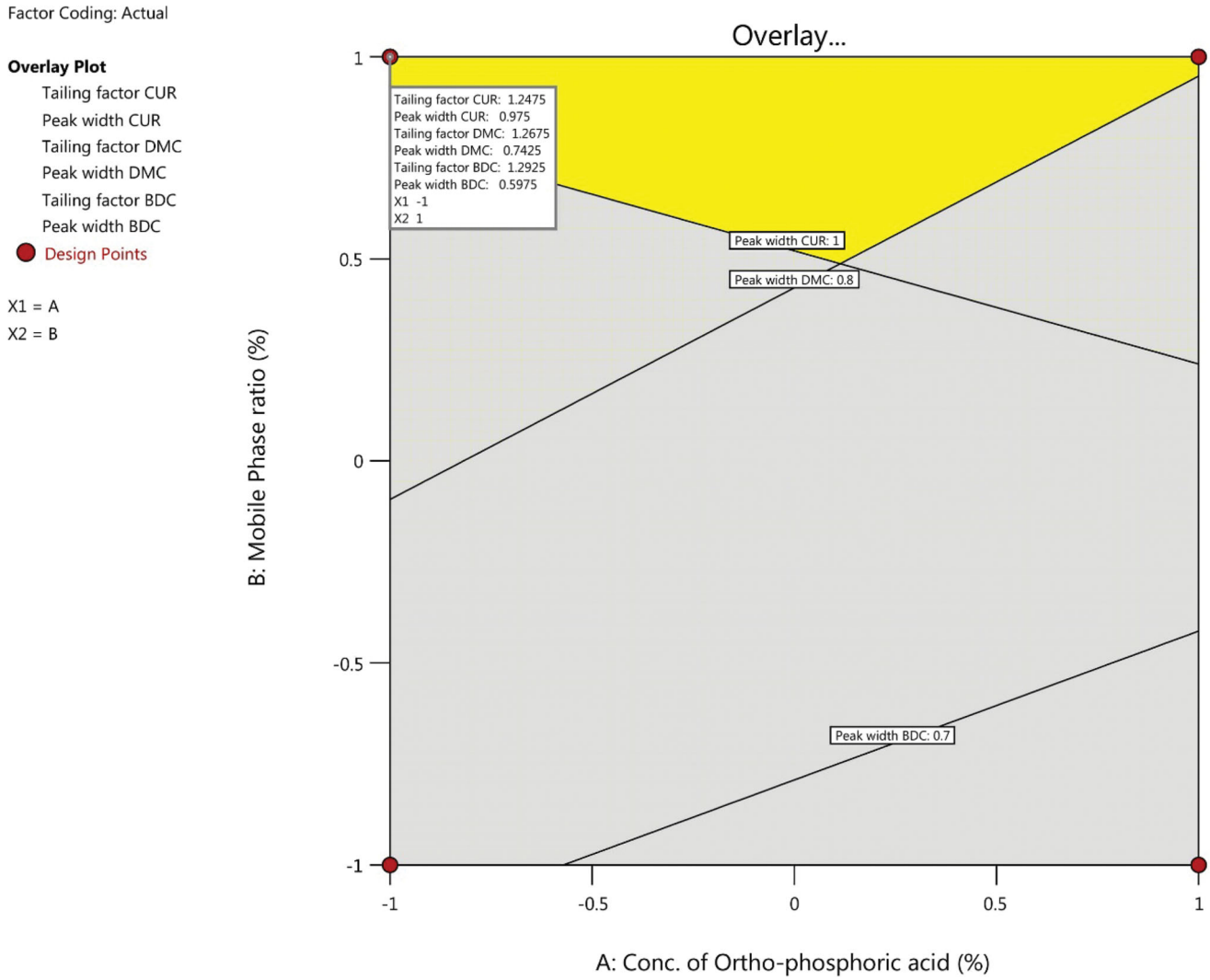

Figure 3. Overlay plot showing the design space with optimal analytical conditions.

summarized in Table 2. Mathematical expressions in the form of polynomial equations were derived which signifies the relationship between the independent variables (Orthophosphoric acid in the aqueous phase (X_1) and mobile phase ratio (X_2)) and responses (Tailing factor CUR (R_1), peak width CUR (R_2), Tailing factor DMC (R_3), peak width DMC (R_4), Tailing factor BDC (R_5), and peak width BDC (R_6)).

A positive sign of coefficient indicates a synergistic effect while a negative term indicates an antagonistic effect upon the response. The larger coefficient means that the independent variable has a more potent influence on the response. To demonstrate graphically the effect of each factor on responses, the Response Surface Plots were generated (Figure 2). The response surface plots provide an overview of the relationship between each dependent variable (CQA's) and independent variables. From the Response Surface Plot indicates blue color with lesser tailing factor; whereas red color indicates greater tailing factor in case of response, R_1 (Tailing factor CUR), R_3 (Tailing factor DMC), and R_5 (Tailing factor BDC). It is evident from Figure 2 that by decreasing the concentration of orthophosphoric acid in the mobile phase tailing factor and peak width decreases.

Table 3. Optimized Chromatographic Conditions.

Parameters	Chromatographic conditions
Stationary phase	ZORBAX C18 (250 mm × 4.6 mm, 5 µ) column
Mobile phase	Acetonitrile:Water (0.02% OPA)
Mobile phase ratio	55:45
Flow rate	1 mL/min
Detection wavelength	425 nm
Injection volume	20 µL
Retention time	
Curcumin	9.86 min
Demethoxycurcumin	9.11 min
Bisdemethoxycurcumin	8.43 min

Establishment of MODR

From the method operable Design region; analytical trial 2 (Conc. of Orthophosphoric acid 0.02% and Mobile phase ratio 55:45) and Trial 3 (Conc. of Orthophosphoric acid 0.05% and Mobile phase ratio 60:40) falls under the region of successful operating ranges and fulfills the criteria of ATP and CQA for HPLC method Figure 3.

The criteria for the selection of optimum run was the ability of chromatographic conditions to yield lesser tailing factor and peak width. Consequently, trial 2 having Conc. of Orthophosphoric acid 0.02% and Mobile phase ratio 55:45 was selected as an optimized HPLC method (Table 3).

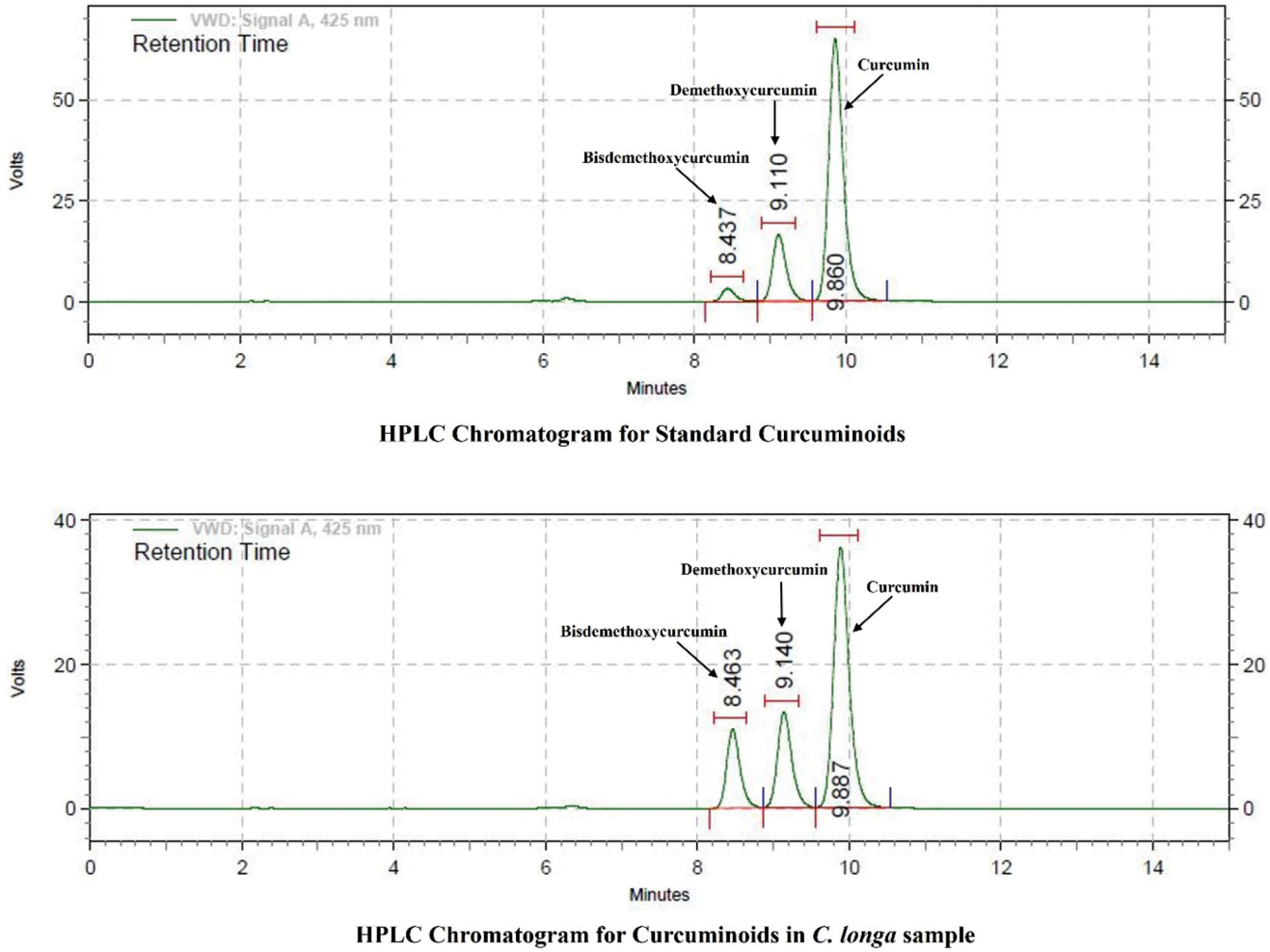

Figure 4. HPLC chromatograms for standard curcuminoids and curcuminoids in *C. longa* crude sample.

Quantification of curcuminoids

The optimized method was further used for quantification of each curcuminoid in crude *C. longa* powdered sample. The quantitative estimation revealed the presence of 13.86 ± 0.28, 10.98 ± 0.23 and 9.32 ± 0.20 mg/g of Curcumin, Demethoxycurcumin and Bisdemethoxycurcumin respectively. The HPLC chromatograms for standard curcuminoids and samples have been depicted in Figure 4.

Method validation

Method validation data for the proposed RP-HPLC method is summarized in Table 4. The system suitability of the developed method was confirmed by the percent RSD of different parameters such as peak area, retention time (Rt), and tailing factor. The percent RSD of peak area, retention time, and tailing factor (<2) were within the acceptable limits. The linear calibration curve for curcumin, Desmethoxycurcumin, Bisdesmethoxycurcumin was obtained for the tested concentration ranges. The determination of LOD and LOQ was done using the data obtained from the linear regression equation of the calibration curve.

The reproducibility and repeatability are the representatives of the Precision of an analytical method. The lower

Table 4. Validation data of the proposed method.

Validation parameters	CUR	DMC	BDC
System suitability			
Retention time			
Mean ± SD	9.86 ± 0.021	9.114 ± 0.016	8.44 ± 0.01
% RSD	0.22	0.17	0.15
Peak area			
Mean ± SD	6,762,417 ± 23,294	1,384,120 ± 12,093	254,234 ± 1189
% RSD	0.34	0.87	0.47
Tailing factor			
Mean ± SD	1.31 ± 0.010	1.30 ± 0.01	1.33 ± 0.01
% RSD	0.79	0.63	0.57
Linearity			
Linearity range (μg/ml)	2–10	2–10	2–10
Slope	118,750	29,401	6354.7
Intercept	115,352	29,546	6159.5
Correlation-coefficient	0.9985	0.9985	0.9981
LOD (μg/ml)	0.45	0.45	0.52
LOQ (μg/ml)	1.38	1.38	1.58
Precision			
Intra-Day (%RSD)	1.38	1.36	1.28
Inter-Day (%RSD)	1.60	1.58	1.35
Accuracy			
50%			
% Recovery	105.21	102.13	99.88
100%			
% Recovery	105.46	102.20	99.15
150%			
% Recovery	101.06	98.48	95.92

intra-day and inter-day % RSD values for Curcumin, Desmethoxycurcumin, and Bisdesmethoxycurcumin respectively indicate the high precision of the developed method. The % recovery was assessed by analyzing the prepared sample at three different concentrations levels, i.e., 50, 100, 150%. The obtained results hence demonstrate a good accuracy of the developed method.

Conclusion

The present research work exemplifies the successful employment of analytical QbD for the development of a simple and robust RP-HPLC method for quantification of Curcuminoids simultaneously. All the quality control parameters performed for assessing the quality of the crude *C. longa* sample represented good results therein confirming the quality and safety of the crude sample. The developed method entails advantages such as reduced analytical time, efficient separation in terms of well-defined peaks, and utilization of simple mobile phase combination. The assistance of QbD principles and DoE tools enabled the detection of the influential factors that were believed to be vital for achieving the most favorable chromatographic conditions for quantification of each curcuminoids accurately. Further, the method was validated in accordance with ICH guidelines. Consequently, it can be concluded that the application of the QbD approach ensured the development of a more robust method that can engender consistent, reliable, and quality data throughout the process and also save time.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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RESEARCH

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Quality assessment and Analytical Quality by Design-based RP-HPLC method development for quantification of Piperine in *Piper nigrum* L.

Vishakha Parab Gaonkar[1*], Vinodh Kumar Mannur[1*] and Kirankumar Hullatti[2]

Abstract

Background: *Piper nigrum* L. is one of the widely used herbs in Ayurvedic medicine. Piperine is a major phytoconstituent that is responsible for most of the activity of the herb. Quality assessment and standardization of such phytoconstituents is the need of the hour. The present study aims at developing a Quality by design (QbD)-based RP-HPLC Method for marker-based standardization of *Piper nigrum* L. fruits along with its quality assessment.

Results: The quality assessment of the crude sample was carried out by evaluating pharmacognostic parameters and analysis of toxic contaminants. The analytical target profile and critical quality attributes were determined and 2^2 factorial design was employed for optimization of the method. By performing the experiments as per the QbD concept the optimized mobile phase was identified as Acetonitrile and Water with 0.05% Acetic acid in the ratio of 70:30, with a flow rate of 1 mL/min and UV detection at 342 nm. The retention time of Piperine was found to be 5.5 min and the amount of Piperine in crude *P. nigrum* fruits and its extract was found to be 3.6% w/w 5.62% w/w, respectively. The Pharmacognostic parameters showed the results within specified limits and the crude drug sample showed the absence of toxic contaminants in it thus indicating the purity of the drug.

Conclusion: The utilization of the QbD approach leads to the development of a more precise and reliable method for the quantification of phytocompounds.

Keywords: *Piper nigrum* L., Piperine, Quality by design, Quality assessment, RP-HPLC

Background

Herbal drugs have been used in medical practice for many years and are gaining considerable momentum in the world during the past decades [1]. As the requirements of herbal drugs are increasing worldwide, their quality control and standardization have become more imperative. Since quality control and standardization of herbal drugs is an important task with great challenges; factors such as geographic and environmental differences of growing conditions, physical constants, adulterations, microbiological contamination, and foreign materials could affect the quality and also batch-to-batch uniformity of herbal products. Hence quality assessment of raw material concerning pharmacognostic and phytochemical parameters is essential in order to prove the identity and purity of herbal drugs [2, 3].

Chemical marker-based standardization is a widely accepted method for the quality control of herbal drugs. In these methods, suitable markers or pharmacologically active compounds in the herb are analyzed by various chromatographic techniques for evaluating the quality and authenticity of herbal medicines. Several chromatographic techniques ranging from simplest Thin Layer Chromatography to sophisticated High-Performance Liquid Chromatography (HPLC), High-Performance Thin Layer Chromatography (HPTLC),

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and Gas Chromatography (GC) can be utilized for such marker-based standardization of herbal drugs [4].

Along with the utilization of marker-based techniques, the application of novel quality approaches are essential for quality assessment and development standardization parameters for herbal drugs. In recent times, pharmaceutical companies adopting Quality by Dsign (QbD) as a fundamental pharmaceutical quality model [5]. Quality by Design is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management" [6]. According to the recent literature the QbD concept can serve as a novel approach for quality control of herbal drugs [7–9].

Application of the QbD approach in analytics is one of the alternatives which reduces the experimental time and cost for drug analysis. The QbD approach suggests looking into the quality of the analytical process during the development stage itself. Analytical QbD explores the scientific understanding of method variables and their interactions, finally provides a region for a highly robust and cost-effective approach [3, 10, 11].

Piper nigrum L. belongs to the family Piperaceae and is known as the Black pepper or king of spices. Black pepper fruits are the source of one of the world's most widely and frequently used spices. The fruits of the plant have a long history of usage in Ayurvedic and folklore medicine, particularly for digestive ailments [12]. The main chemical constituents of fruits are alkaloids, among the alkaloid content Piperine is a major phytoconstituent that is responsible for most of the activity of the herb. In Ayurvedic medicine, black pepper has been used to aid digestion, improve appetite, treat coughs, colds, breathing and heart problems, colic, diabetes, anemia, and piles. It improves drug availability and is used as a bio enhancer due to its ability to enhance the efficacy of other drugs [13, 14]. In the present research work, an attempt has been made to develop quality control standards for *P. nigrum* fruits by carrying out the pharmacognostic evaluation along with chemical marker-based standardization of *P. nigrum* fruits by application of the Analytical QbD approach.

Methods

Chemicals

Standard Piperine was provided as a gift sample by the Himalaya drug company, Bengaluru India. Acetonitrile and water of HPLC grade purchased from Merck, Mumbai, India Pvt Ltd. Other chemicals and reagents used in the research work were of analytical grade.

Plant material and processing

Sample of crude *Piper nigrum* L. fruits was procured and authenticated from Shri B. M. Kankanwadi Ayurveda Mahavidyalaya, Karnataka. The fruits were shade dried and were coarsely ground into homogenous powder using a mechanical grinder and stored at room temperature. The extraction of the crude drug was carried out by cold maceration followed by the soxhlet extraction method. Ethanol and Water in the ratio of 90:10 was used for the extract preparation.

Quality assessment of *P. nigrum* fruits

The quality of *P. nigrum* fruits was assessed by evaluating the quality control parameters mentioned in WHO guidelines. Physico-chemical parameters including moisture content, extractive value, and ash value were performed along with phytochemical analysis [15, 16]. The crude sample of *P. nigrum* fruits were further analyzed for the determination of toxic substances such as Aflatoxins, pesticide residues, and heavy metals. Aflatoxins were determined by HPLC method as per the standard procedure [17]. Aflatoxins B1, B2, G1, and G2 were analyzed in the powdered sample. Analysis of pesticide residue was carried out by Gas Chromatography–Mass spectroscopy (GC–MS) Instrument. The presence of a total of 17 pesticide contaminants was analyzed in crude powdered fruits. And the presence of heavy metals was analyzed by Atomic Absorption Spectroscopy. Heavy metals, namely lead, cadmium, arsenic, mercury, and chromium were tested in the crude powdered sample.

Instrumentation and chromatographic conditions

HPLC system (Agilent technologies 1220 Infinity II LC) used for the analysis consisted of a system controller, low-pressure gradient pump, solvent delivery module, online degasser, manual sample injector (injection volume ranging between 5 and 20 µL), and UV–Vis detector. A Reversed-phase C-18 column (5 µm, 4.6 mm, 250 mm, ZORBAX) was used for chromatographic separation. The mobile phase was composed of acidic Water adjusted with Acetic acid, and Acetonitrile in different ratios. Samples were analyzed at the flow rate of 1 mL/min and the detection wavelength was set at 342 nm. For each analysis, a 20 µL sample was injected into the column.

Preparation of standard and sample solution

A stock of solution of standard Piperine (1 mg/mL) was prepared by dissolving accurately weighed 10.00 mg of Piperine in 10.00 ml of HPLC grade methanol with the help of a sonicator. Further working standard solutions

were prepared by diluting the stock solution with the mobile phase.

For the preparation of the sample, accurately weighed 10 mg of crude powdered sample and extract of *P. nigrum* fruits was transferred to 10.00 mL volumetric flask individually containing 5.00 mL of methanol. The methanolic solution was sonicated for 15.00 min to ensure the complete dissolution of piperine. The volume was made up to 10.00 mL with methanol and was used for further analyses. The solution was filtered through a 0.25 µm membrane filter prior to their injection into the chromatographic column.

Analytical Quality by design assisted based HPLC method development

Defining of analytical target profile (ATP) and critical quality attributes (CQA)

Defining of Analytical Target Profile (ATP) is the first step in the Analytical QbD. ATP serves as the quality specification of the analytical method which should be achieved so as to attain reliable results. Critical Quality Attributes (CQA's) are the quality characteristics related to method performance. CQAs have to be identified from the defined ATP which will be useful in ascertaining the satisfactory performance of the developed method [10, 18].

Optimization of method using design of experiments (DoE)

The optimization of the analytical method was typically performed on parametric variables using Design of Experiments (DoE) to ensure that maximum understanding is gained while minimizing the total number of experiments. A simple 2^2 full factorial design with 2 factors and 2 levels, resulting in 4 experimental runs was employed in order to identify the optimized analytical conditions. The DoE was developed using Design-Expert software version 12.0, (Stat-Ease Inc., Minneapolis, MN, USA).

Establishment of method operable design region (MODR)

After performing the experimental runs planned as per 2^2 factorial design, the obtained data was studied in terms of regression models and factor-response relationship, to generate the Method Operable Design Region (MODR). From the established MODR the optimized chromatographic conditions were predicted based on the specified target or goals of each CQA in terms of overlay plot. Within the design region, all the specifications mentioned in the ATP are fulfilled at a specified risk level.

Validation of the optimized method

Validation of the optimized RP-HPLC method was performed as per ICH Q2 (R1) guidelines [19]. The described method was extensively validated in terms of system suitability, linearity, LOD, LOQ, Intra-day precision, Inter-day precision, and accuracy.

Results

Quality assessment of *P. nigrum* fruits

Quality assessment of herbal drugs is one of the most essential and crucial tasks which enables the determination of quality and safety of the crude drug. Evaluation of physicochemical properties serves as a tool for quality control and identification of crude drugs. Physicochemical parameters such as the moisture content, aqueous soluble extractive value, alcohol soluble extractive value, total ash value, acid insoluble ash value, and water-soluble ash value was found to be 7.6 ± 0.69, 8.67 ± 0.58, 9.83 ± 0.76, 3.83 ± 0.29, 0.45 ± 0.21, and $2.67 \pm 0.29\%$ w/w, respectively.

The preliminary phytochemical analysis gives a brief idea about the presence of various secondary metabolites in medicinal plant materials. The preliminary phytochemical analysis of *P. nigrum* fruits revealed the presence of important secondary metabolites such as alkaloids, flavonoids, tannins, and steroids whereas phenols, saponins, and glycosides were found to be absent.

Determination of toxic substances in herbal drugs is one of the major criteria for the assessment of quality in herbal drugs. Determination of Aflatoxins, heavy metals, and pesticide residue is a prerequisite criterion for the export of herbal drugs to foreign countries. Hence, this serves as a vital measure for defining the quality of herbal drugs or products [20]. The HPLC chromatogram obtained from the Aflatoxins analysis in the sample is depicted in Fig. 1. Our earlier study has

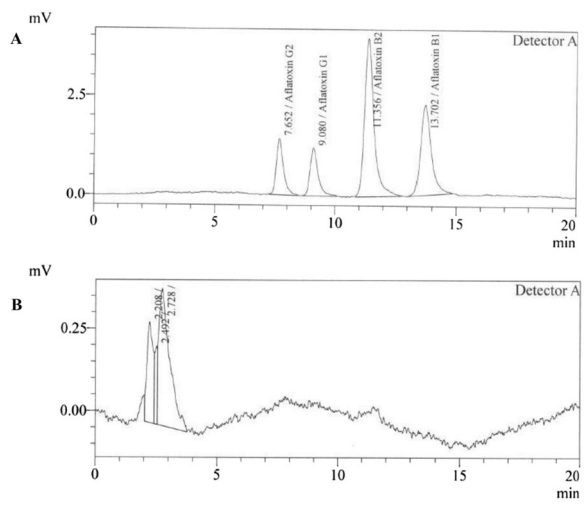

Fig. 1 HPLC chromatograms for (**A**) standard aflatoxins and (**B**) *P. nigrum* sample

reported the retention time for Aflatoxins standards B1, B2, G1, and G2 as 13.70, 11.35, 9.08, and 7.65 min, respectively [11]. From the sample chromatogram, it can be observed that no significant peaks are obtained at the above mentioned retention times, indicating the absence of Aflatoxins in the crude *P. nigrum* sample. Further, the pesticide residues and heavy metals in the sample were found to be below the limit of quantification. Where, the quantification limit for pesticide residues, lead, cadmium, mercury, arsenic and chromium being 0.01 mg/kg, 1.1 mg/kg, 0.5 mg/kg, 0.1 mg/kg, 0.1 mg/kg, and 0.5 mg/kg, respectively.

Initial method development

Initial chromatographic conditions were selected based on a thorough literature survey particularly relating to the physicochemical properties of the piperine such as its pKa, solubility, acidic nature, etc. most of the previously published studies have used organic solvents such as methanol, acetonitrile, etc. along with aqueous phase adjusted to high acidic pH and buffer solutions [21–27]. Further utilization of different column conditions, flow rate, and wavelength has also been observed. Moreover, the previously reported literature exhibits peaks with poor resolution or increased retention time. Taking a gist from the previous studies several preliminary trials were conducted, amongst them a mobile phase consisting of a systemic composition of Acetonitrile, water, and acetic acid as the pH modifier with suitable flow rate and wavelength was used for the quantitative estimation of piperine.

QbD based RP-HPLC method development

The predetermined quality characteristics that are known to enhance the method performance are referred to as Analytical target profile. The selection of ATP is purely dependent on the quality attributes that we want in the method. Defining of ATP is the primary step of AQbD concept. TheATP of the proposed analytical method is to achieve a good separation for quantification of Piperine, with lesser tailing factor and peak width along with acceptable analysis time. Based on the above-mentioned ATP, CQAs were identified as Tailing factor (Not more than 2) and Peak width (Not more than 2).

Method optimization by DoE

As per the adopted per 2^2 full factorial design, two independent variables, i.e., % concentration of Acetic acid in aqueous phase (X1) and mobile phase ratio (X2) were varied at two different levels that were coded for low and high (−1 and +1 respectively). Tailing factor (R1) and peak width (R2) were selected as the dependent or response variables. The DoE software was used to gain information on the critical values required to achieve the desired response of the selected independent variables.

The response, tailing factor (R1) and peak width (R2) obtained for each chromatographic trial are summarized in Table 1. Further, statistical optimization of the analytical method was performed by comparison of several statistical parameters, provided by Design-Expert® Software, Version 12. The statistical data of the applied design is summarized in Table 2. The relationship between the selected independent and dependent variables was derived by studying the mathematical

Table 1 Selected factor combinations for Piperine as per 2^2 full factorial design

Code	Coded levels		Actual values		Responses	
	X1	X2	X1 (%)	X2	R1	R2
T1	−1	−1	0.05	60:40	1.31	0.49
T2	−1	+1	0.05	70:30	1.23	0.43
T3	+1	+1	1	70:30	1.47	0.55
T4	+1	−1	1	60:40	1.57	0.62

X1-conc. of acetic acid (%); *X2*- mobile phase ratio, *R1*-tailing factor, *R2*- peak width

Table 2 Summary of statistical parameters and polynomial equation

Response	*P* value	Model significance	Polynomial equation
Piperine			
R1	0.0376	Significant	+1.39 + 0.1250 * X1 − 0.0450 * X2
R2	0.0355	Significant	+0.5225 + 0.0625 * X1 − 0.0325 * X2

X1 and X2 are independent variables where, X1—conc. of acetic acid in aqueous phase and X2—mobile phase ratio

expression in the form of polynomial equations. A positive sign of coefficient indicates a synergistic effect while a negative term indicates an antagonistic effect upon the response. The larger coefficient means that the independent variable has a more potent influence on the response. Graphical demonstration in the form of Response Surface Plot was generated (Fig. 2) to understand the effect of each factor on responses. The response surface plots provide an overview of the relationship between each dependent variable (CQA's) and independent variables. Response Surface Plot shows the colored regions from blue to red indicating the intensity of the responses from lower to higher end. From the plot, it is observed that by decreasing the level of variable X1, i.e., concentration of acetic acid in the aqueous phase, the value of both the responses R1and R2 decreases which means that by decreasing the concentration of acetic acid in the aqueous phase tailing factor and peak width decreases. This further indicates that variable X1 has a significant impact on response R1 and R2 when compared to variable X2.

Establishment of MODR

Method operable design region (MODR) is a multidimensional combination and interaction of independent factors which further lead to the selection of acceptable operating ranges that assure quality. Figure 3 shows MODR (overlay plot) with the optimum region as a design space in yellow shade and selected method conditions were represented using flag. From the method operable Design region, analytical trial T1 (Conc. Of Acetic acid 0.05% and Mobile phase ratio 60:40) and T2 (Conc. Of Acetic acid 0.05% and Mobile phase ratio 70:30) falls under the region of successful operating ranges and fulfills the criteria of ATP and CQA for HPLC method. Among both, the trials T2 having Conc. Of Acetic acid 0.05% and Mobile phase ratio 70:30 was selected as optimized HPLC method due to its ability to give lesser tailing factor and peak width (Table 3).

Quantitative estimation of Piperine

The optimized method was further used for the quantification of Piperine in crude *P. nigrum* fruits as well as in the extract. The quantitative estimation revealed the presence of 3.6%w/w and 5.62%w/w of Piperine in crude powder and extract respectively. The HPLC chromatograms for standard Piperine and samples have been depicted Fig. 4.

Method validation

The developed RP-HPLC method was validated to confirm its suitability for its intended purpose as described in ICH Q2 (R1) guidelines. The validation parameters of the proposed RP-HPLC method are summarized in Table 4 which was found to be within the standard limits specified in ICH Guidelines.

The system suitability of the developed method was confirmed by the percent Relative Standard Deviation (RSD) of different parameters such as peak area, retention time (Rt), and tailing factor. The percent RSD of peak area, retention time, and tailing factor (<2) were within the acceptable limits. The linear calibration curve for piperine was obtained for the selected concentration range (Fig. 5). The LOD and LOQ were determined from the linear regression data obtained from the calibration curve. The reproducibility and repeatability indicate the Precision of an analytical method. The lower intra-day

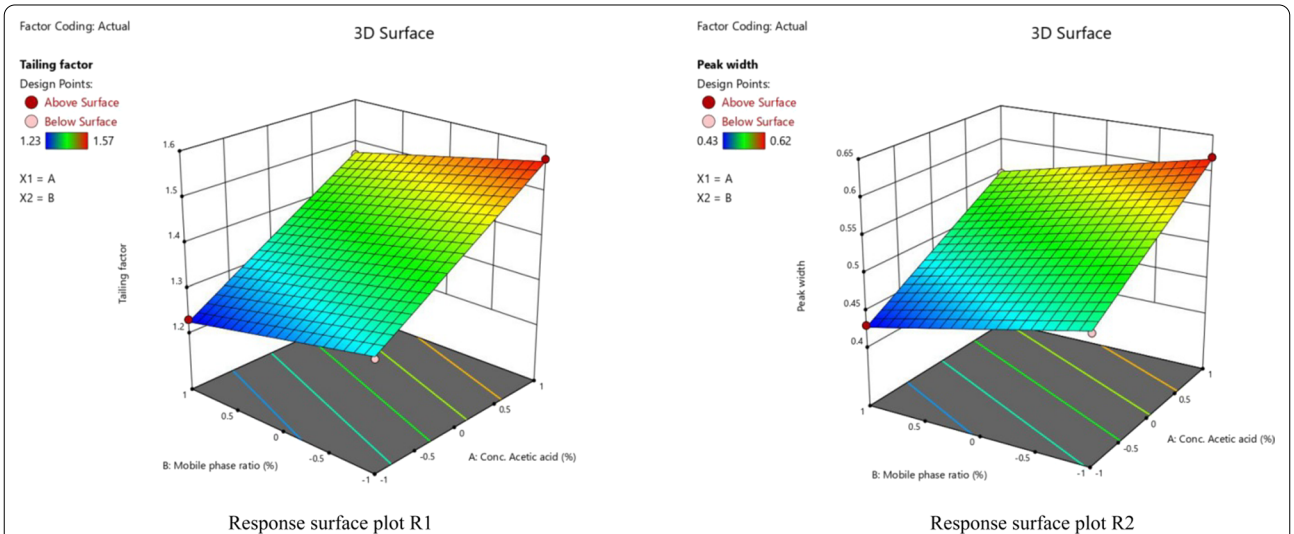

Fig. 2 Response surface plot for optimization of HPLC method for Piperine

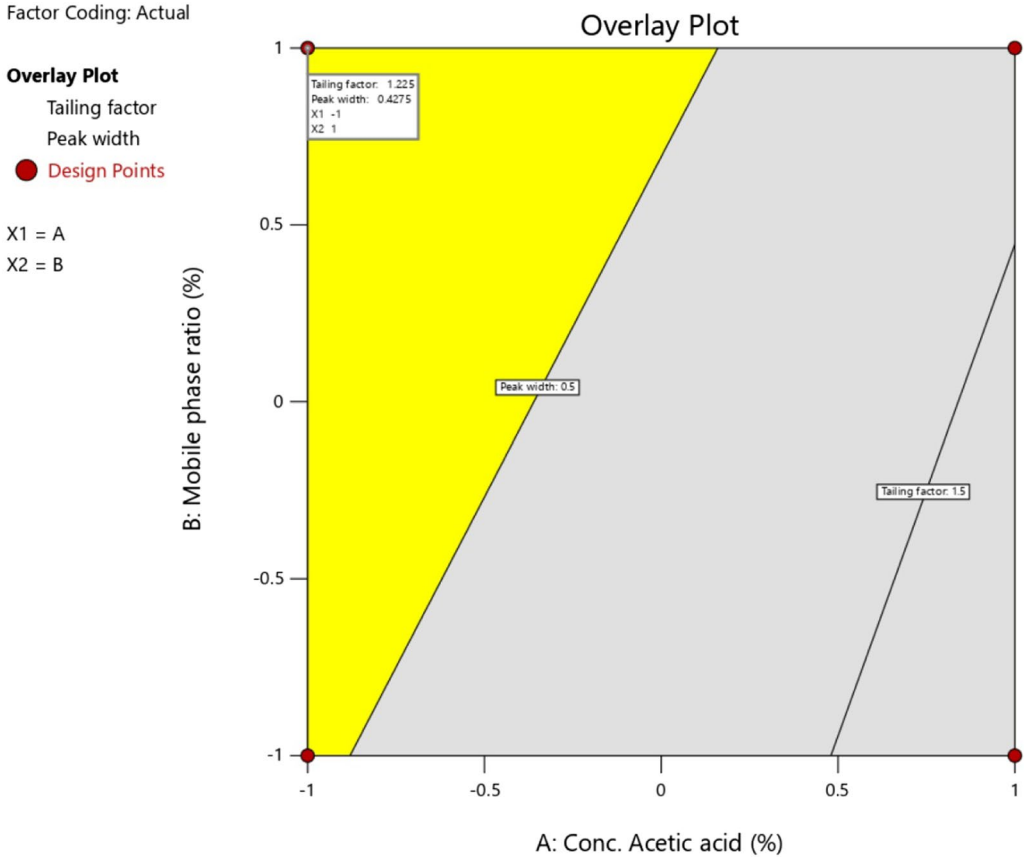

Fig. 3 MODR for optimization of HPLC method for Piperine

Table 3 Optimized chromatographic conditions

Parameters	Chromatographic conditions
Stationary Phase	ZORBAX C-18 (250 mm × 4.6 mm, 5 µ) column
Mobile phase	Acetonitrile:Water (0.05% acetic acid)
Mobile phase ratio	70:30
Flow rate	1.00 mL/min
Detection wavelength	342.00 nm
Injection volume	20.00 µL
Retention time	5.5 min

and inter-day % RSD values for piperine demonstrated the high precision of the developed method. The % recovery of the piperine obtained from the sample indicates a good accuracy of the developed method.

Discussion

The present research work was carried out to endeavor the development of Analytical Quality by design-assisted RP-HPLC method for estimation of Ppiperine in *P.nigrum* fruits. Though many studies have been conducted on the RP-HPLC method for analysis of Piperine, our study stands out amongst them as it reports the utilization of the Analytical QbD concept. With the help of AQbD principles, a suitable ATP has been developed which serves as a quality specification guide for the development of the analytical method. Based on the ATP, Critical Quality Attributes were identified. By taking into consideration the critical analytical parameters such as concentration of acids used in mobile phase and mobile phase ratios experiments were designed by DoE tools. Here, the CQAs were thoroughly examined by performing statistical analyses such as ANOVA. Polynomial equations and 3-D response surface plots were also developed for identifying the relationship between analytical parameters and CQAs. Further, the optimized chromatographic conditions were predicted from the MODR exhibiting yellow shaded region of successful operating ranges. The optimized chromatographic condition was then applied for quantitative estimation of Piperine in crude and extracted *P. nigrum* fruits which were found to be 3.6%w/w and 5.62%w/w, respectively. Apart from the development of AQbD assisted RP-HPLC method our study also reports the data on the Quality

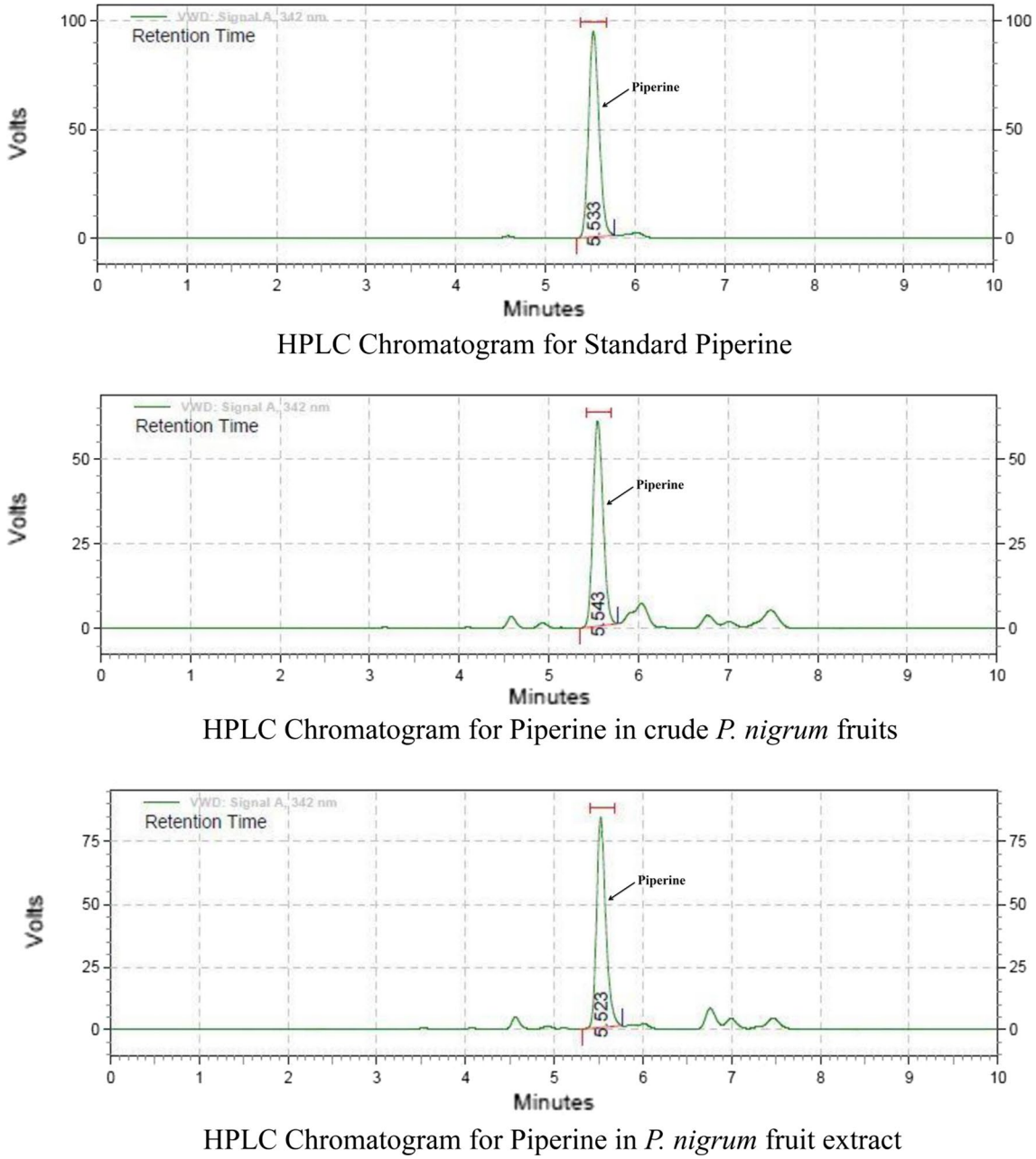

Fig. 4 HPLC chromatograms for standard Piperine, crude *P. nigrum* L. fruits and extract

assessment of *P. nigrum* fruits with special reference to the estimation of toxic substances found in herbal crude drugs. The study has also represented the Phytochemical and Physico-chemical characteristics of the *P. nigrum* fruits. Valuable data on the determination of Aflatoxins, pesticide residues, and heavy metals have been reported which is mainly considered a prerequisite by regulatory authorities for ensuring the quality and safety of herbal drugs.

Conclusions

In the present research work, AnalyticalQbD-assisted RP-HPLC method was developed and validated for the quantitative estimation of Piperine in *P. nigrum* fruits and its extract. ATP and CQAs for the proposed method was outlined with the execution of chromatographic trials as per 2^2 full factorial design. Based on the obtained Design space, optimized chromatographic conditions were predicted. Furthermore, the crude *P. nigrum* was

Table 4 Summary of validation parameters

Validation parameters	Piperine
System suitability	
Retention time	
Mean ± SD	5.54 ± 0.003
% RSD	0.05
Peak area	
Mean ± SD	20,156,202 ± 120,229
% RSD	0.59
Tailing factor	
Mean ± SD	1.24 ± 0.01
% RSD	0.68
Linearity	
Linearity range (µg/mL)	5.00–25.00
Correlation-coefficient	0.9986
LOD (µg/mL)	1.12
LOQ (µg/mL)	3.41
Precision	
Intra-day (%RSD)	1.35
Inter-day (%RSD)	1.56
Accuracy	
80%	
% Recovery	99.85 ± 0.28
100%	
% Recovery	101.20 ± 0.02
120%	
% Recovery	96.35 ± 0.05

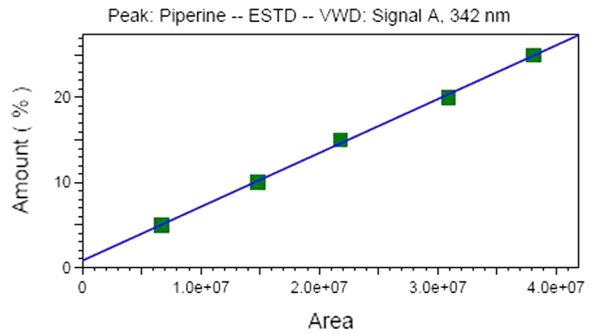

Fig. 5 Linear calibration curve for Piperine

also extensively evaluated for quality control parameters, which was useful for ascertaining the quality of the herbal drug under study. Employment of the QbD tools has helped in developing a chromatographic method that has the potential of providing reproducible and reliable results along with the reduction in analysis time and cost. From the above findings, we can affirm that the application of the QbD approach for the standardization of herbal drugs can serve as an important tool for quality standardization of herbal drugs.

Abbreviations

QbD: Quality by Design; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; ATP: Analytical Target Profile; CQA: Critical Quality Attributes; DoE: Design of Experiments; MODR: Method Operable Design Region; LOD: Limit of Detection; LOQ: Limit of Quantification.

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Authors' contributions

VPG carried out the experimental work. VSM and VPG equally contributed in framing and writing of manuscript. KKH revised and edited the final manuscript file. All authors have read and approved the manuscript.

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Availability of data and material

The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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REVIEW ARTICLE

Indian Traditional medicinal plants as a source of potent Anti-diabetic agents: A Review

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Abstract

Objective The present review aims to provide an overview of traditional medicinal plants known to be of anti-diabetic potential.

Methods A literature search was conducted using the scientific databases including PubMed, EMBASE and google scholar and a total of fifty herbs have been described and their possible mechanism of anti-diabetic action has been mentioned. Among them, in-depth discussion on five most potent anti-diabetic herbs has been provided with respect to their mechanism of action, in-vivo studies and clinical efficacies.

Results The present review has highlighted the usefulness of the herbal source for the treatment and management of diabetes mellitus. With the help of previous literature published on *In-vivo* animal studies and human clinical studies; the effectiveness of *Gymnema sylvestre, Momordica charantia, Trigonella foenum graecum, Tinospora cordifolia and Curcuma longa* in the treatment and management of Diabetes has been proved.

Conclusion Based on this review it can be concluded that herbs can serve as more efficient, safer, and cost-effective adjuvant therapy in the management and treatment of diabetes. Further investigations mainly focusing on the isolation of phytocompounds from these herbs can lead to the discovery of newer antidiabetic agents.

Keywords Anti-diabetic · Diabetes mellitus · Herbs · Hyperglycaemia · Medicinal plants · Phytocompounds

Introduction

Diabetes mellitus is one of the most prevalent diseases found in all parts of the world and is becoming a serious threat to mankind's health [1]. It is a complex heterogeneous group of metabolic disorders including hyperglycemia and is associated with the imbalance in carbohydrate, protein, and lipid metabolism [2]. According to WHO, "Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the blood, which in turn damage many of the body's systems, in particular the blood vessels and nerves" [3]. According to the recent data by International Diabetes Federation (IDF) Atlas claims that around 463 million adults are currently living with diabetes and estimates that there will be 578 million adults with diabetes by 2030, and 700 million by 2045 [4].

The management of diabetes mellitus is considered a global problem. In current allopathic therapy the oral hypoglycaemic agents and insulin, are subsequently used to control the diabetic conditions, however, complications associated with them, limited tolerability, cost, and other side effects reduce its wide acceptance. This could be the main reason for the shift of common people to Ayurveda form allopathic system nowadays [5].

Since ancient times traditional herbal drugs with multiple phytoconstituents and properties have been used as medicines for the treatment of a wide range of diseases [6]. Herbal medicines have been considered to be intrinsically safe, due to their natural occurrence, efficacy, and fewer side effects [7]. India has a long history of use of medicinal plants for the management of diabetes. World ethnobotanical information has reported the usage of about 800 plants for the control of diabetes mellitus, amongst them only 410 are experimentally proven for having anti-diabetic properties but the complete mechanism of action is available only for about 109 plants

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[8]. The treatment of diabetes using herbs has more advantageous effects and does not cause much side effects. These herbal drugs act by different mechanisms and consequently protect the β-cells during the diabetic condition and reduce the amount of glucose level in the blood [9].

This review aims to provide an overview of the use of medicinal plants in the management of diabetes, focusing on their mechanism of action. Furthermore, an emphasis on the five most commonly available and potent anti-diabetic herbs has been given. These include *Gymnema sylvestre*, *Momordica charantia*, *Trigonella foenum graceum, Tinospora cardifolia*, and *Curcuma longa*.

Methods

A literature search was conducted using the scientific databases including PubMed, EMBASE and google scholar. The aim was to identify published data on traditionally used medicinal plant for the treatment and management of Diabetes mellitus. The search terms used were "diabetes and plants", "traditional plants", "medicinal plants and diabetes", "anti-hyperglycemic plants", and "mechanism of anti-diabetic action". Based on the above criteria extensive literature search was carried out and a total of fifty herbs have been described with their possible mechanism of anti-diabetic action. Amongst the fifty herbs in-depth discussion on five most potent and easily available anti-diabetic herbs has been provided with respect to their mechanism of action, in-vivo studies and clinical efficacies.

Traditional anti-diabetic plants

Since the time of Charaka and Sushruta, traditional medicines have been used for the management of diabetes mellitus [10]. Medicinal plants have always been a valuable source of drugs and many of the currently available drugs such as aspirin, quinine, vincristine, vinblastine, and digitalis have been derived directly or indirectly from them [11]. Most of the anti-diabetic drugs derived from plants are from the phytochemical class of polyphenols, terpenoids, tannins, and steroids. These affect various metabolic cascades, which further affect the level of glucose in the human body [12].

A list of medicinal plants used traditionally for diabetes with proven anti-diabetic and related beneficial effects are compiled along with their family, active principles responsible for diabetes, mechanism of action and use (Table 1).

Amongst the fifty herbs described in Table 1 we have identified five most potent and easily available anti-diabetic herbs namely, *Gymnema sylvestre, Momordica charantia, Trigonella foenum graecum, Tinospora cordifolia and Curcuma longa*. In-depth discussion on these herbs has been provided with respect to their mechanism of action, in vivo studies and clinical efficacies. The rationale behind selection of these five herbs owes to their traditional usage, easy availability, effectiveness and most importantly to their proven clinical significance. The selected plants are a choice of herbal medicine in the treatment of diabetes. They are also utilized by multiple pharmaceutical herbal industries and most of the herbal anti-diabetic preparation consist of these herbs. Although there are other plants which are utilized in the diabetes management as folklore medicine but their commercial utilization is less as compare to the chosen five plants. Hence a need has been felt for understanding their various mechanism of actions and efficacy in management of diabetes.

Gymnema sylvestre

Gymnema sylvestre is an indigenous herb, belonging to the family Asclepiadaceae. It is popularly known as "gurmar" for its distinct property as sugar destroyer, it is a reputed herb in the Ayurvedic system of medicine. The plant is indigenous to western and central India, Australia, and tropical Africa [94].

Phytochemistry of *G. sylvestre*

G. sylvestre is a good source of a large number of bioactive substances. The leaves contain Triterpene saponins like gymnemic acids, gymnemasaponins, and gymnemasides. Apart from this, other phytoconstituents include flavones, anthraquinones, pentatriacontane, hentri-acontane, α and β-chlorophylls, phytin, stigmasterol, dquercitol, resins, etc. The major secondary metabolites present in Gymnema includes Gymnemic acid. The Gymnemic acids consist of numerous members termed as gymnemic acids I–VII, gymnemasaponins, and gymnemosides A–F. Gurmarin is another essential phytoconstituent isolated from *G. sylvestre* [95].

Mechanism of Action

Antidiabetic activity of Gymnemic acids appears to be due to a combination of mechanisms. It acts through stimulation in insulin secretion from the pancreas. It also shows a similar effect by delaying the glucose absorption in the blood. In the intestine it attaches to the receptor present in the external layer of the intestine, thereby preventing the absorption of sugar molecules by the intestine, resulting in low blood glucose levels [95]. In a study extract of the plant has showed its effectiveness in regeneration of pancreatic β cells [66]. Gymnemic acid the major phytocompounds present in the plant is reported to interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis pathway [96]. Moreover, *G. sylvestre* has been reported to exhibit significant inhibitory activity against α-glucosidase; Fig. 1.

Table 1 List of Traditional plants used in management and treatment of Diabetes

Plant name	Family	Parts used	Active Principles	Mechanism of action	Uses	Reference
Acacia arabica	Leguminoceae	Bark	Gallic acid, pyrocatechol, (+)-catechin, (-) epigallocatechin-7-gallate, (-) epicatechin, quercetin, (+) catechin-5-gallate.	Act as secretagouge to release insulin	Hypoglycemic activity	[13, 14]
Achyranthes aspera	Amaranthaceae	Leaves, seeds.	Betaine, achyranthine, β ecdysone	Carbohydrate digestion and absorption	Hypoglycemic effect	[15, 16]
Adhatoda vasica	Acanthaceae	leaves	Vasicine Vasicinol	α-Glucosidase-inhibiting activity	Antidiabetic	[17, 18]
Aegle marmelose	Rutaceae	leaves	Aegelin, marmesin and marmelosin	Regeneration of pancreatic β cells and insulin secretion	Hypoglycaemic effect	[16, 19]
Ageratum conyzoides	Asteraceae	leaves	Mono- and sesquiterpenes	Increase peripheral utilization of glucose	Hypoglycaemic effect	[20, 21]
Allium cepa	Amaryllidaceae	bulb	S-methyl cysteine sulfoxide, S-allyl cysteine sulfoxide	Stimulates pancreatic β-cells	Hypoglycaemic effect	[22, 23]
Allium sativum	Amaryllidaceae	bulb	Allicin, apigenin, alliin	Stimulates pancreatic β-cells	Antidiabetic and anti-oxidant	[16, 23, 24]
Aloe barbadensis	Asphodelaceae	leaves	Aloin, barbaloin, isobarbaloine, aloetic acid.	Insulin secretion and synthesis	Hypoglycemic effect.	[16, 25]
Andrographis paniculata	Acanthaceae	Whole plant	Andrographolide,	Regeneration of pancreatic β cells, insulin secretion	Antidiabetic & hepatoprotective.	[26, 27]
Annona squamosa	Annonaceae	leaves	Acetogenin	Enhances insulin level from pancreatic islets, increased utilization of glucose in muscle.	Hypoglycemic and antihyperglycemic activities	[28, 29]
Areca catechu	Palmitaceae	Leaves, flowers, seeds	Nitrosamines, arecoline, arecaidine	Carbohydrate digestion and absorption	Hypoglycemic	[16, 30]
Azadirachta indica	Meliaceae	leaves	Azadirachtin, nimbolinin, nimbin, nimbidin, quercetin.	Improves the insulin signaling molecules and glucose utilization in the skeletal muscle.	Antidiabetic, Antibacterial, antioxidant	[31, 32]
Bacopa manneri	Serophulariacea	Aerial part	Bacosine, brahmine, bacopaside I, II, III, IV and V.	Increase in peripheral glucose consumption	Antihyperglycemic agent	[33, 34]
Bauhinia forficuta	Fabaceae	leaves	Kaempferitrin	Glycolysis, insulinonematic activity.	Hypoglycemic effect, antioxidant.	[35, 36]
Berberis aristata	Berberidaceae	Stem bark, roots, leaves	Barberin,	Glucose transport, carbohydrate digestion and absorption, DPP-IV inhibition	Hypoglycemic effect	[37, 38]
Boerhavia diffusa	Nyctaginaceae	leaves	Punarnavine, Boeravinone A-F	Increase in hexokinase activity, increase plasma insulin level, antioxidant	Antidiabetic	[39, 40]
Camellia sinensis	Thecaceae	leaves	Epigallocatechin-gallate, gallocatechin, epicatechin, (+) catechin, (−) epicatechin	Free radical scavenging activity, insulinonematic activity	Antihyperglycemic activity, antioxidant	[41, 42]
Caseria esculenta	Salicaceae	roots	Leucopelargonidin, Dulcitol, Beta sitosterole.	Insulin secretion	Antihyperglycemic activity	[43]
Cassia auriculata	Fabaceae	roots	Bis (2-ethyl hexyl) phthalate	α-Glucosidase-inhibiting activity	Antihyperglycemic effect	[44, 45]
Centella asiatica	Apiaceae	Whole plant	asiaticoside		Antihyperglycemic activity	[46, 47]
Coccinia indica	Cucurbitaceae	Aerial parts	- β- Amyrin Acetate, Lupeol, Cucurbitacin B, Taraxerone, Taraxerol, β-carotene, Lycopene,	Initiate insulin secretion, carbohydrate digestion and absorption.	Hypoglycemic effect.	[48, 49]
Commelina communis	Commelinaceae	Leaves, stem	1-deoxynojirimycin, (2*R*,3*R*,4*R*,5*R*)2,5-bis(hydroxymethyl)-3,4-dihydroxypyrrolidine	Inhibition of α-glucosidase	Antihyperglycemic agent.	[50, 51]
Curcuma longa	Zingiberaceae	rhizomes	Curcumin, termerone, germacrone, zingiberene	Inhibition of α-glucosidase, inhibition of GSK-3β	Antidiabetic, Antihyperlipidemic, antioxidant	[52, 53]
Cyprus rotandus	Cyperaceae	Whole plant	α cyperone, cyperene, cyperol.	Inhibites intestinal glucose absorption and promoting glucose consumption.	Hypoglycemic agent	[54, 55]
Emblica officinalis	Euphorbiaceae	fruits	Gallic acid, ellagic acid, vitamin c.	Hypoglycemic, Decreases lipid peroxidation, antioxidant.	Hypoglycemic and antioxidant.	[56, 57]
Enicostema littorale	Gentianaceae	Whole plant	Swertiamarin, apigenin, isovitexin, swertisin, saponarin, 5-o glucosylswertisin	Glucose-induced insulin release through K(+)-ATP channel.	Hypoglymcemic effect.	[58, 59]

Table 1 (continued)

Plant name	Family	Parts used	Active Principles	Mechanism of action	Uses	Reference
Ficus benghalensis	Moraceae	Bark, leaves	Leucocyanidin, pelarogonidin	Insulin secretion, glycogen synthesis	Antidiabetic	[60, 61]
Ficus racemosa	Moraceae	Bark, leaves	β-sitosterol, racemosic acid, Bergenin.	Glycogenolysis and gluconeogenesis	Hypoglycemic activity	[62, 63]
Glycyrrhiza glabra	Leguminoceae	roots	Glycyrrhizin, glycyrrhizic acid liquirtin, isoliquirtin.	Potent PPAR-γ ligand binding activity thus, reduces the blood glucose level	Hypoglycemic agent.	[64, 65]
Gymnema sylvestre	Asclepidaceae	leaves	Gymnenic acid, Stigmasterol, Gurmarin, betaine, gymnemosides.	Regeneration of pancreatic β cells, α-glucosidase inhibitor, insulin secretion	Antidiabetic agent.	[66–68]
Ginkgo biloba	Ginkgoceae	leaves	Kaempferol, isorhamnetin	Inhibition of α-amylase and α-glucosidase activity	Hypoglycemic agent.	[69]
Mangifera indica	Anacardiaceae	leaves	Mangiferin	α-Glucosidase-inhibiting activity	Hypoglycemic agent	[70]
Momordica charantia	Cucurbitaceae	fruits	Momordin, momordicine, charantin	Insulin secretion, glycogen synthesis	Hypoglycemic agent	[71, 72]
Morus indica	Moraceae	leaves	Chrysin, isoquercitrin	Insulin secretion	Hypoglycemic agent	[73]
Ocimum sanctum	Lamiaceae	leaves	Eugenol, trans-β ocimene, Carvacrol, linalool.	Insulin secretion, carbohydrate digestion and absorption	Hypoglycemic agent.	[74, 75]
Panax ginseng	Araliaceae	roots	Ginsenosides Rg2, panaxan A, B, C, D, E	Regeneration of pancreatic β cells, free radical scavenging	Antihyperglycemic activity	[76, 77]
Phyllanthus amarus	Phyllanthaceae	leaves	Brevifolin carboxylic acid, ethyl brevifolin carboxylate	α-Amylase inhibitory activity	Hypoglycemic, Anti-oxidant activity.	[78]
Pterocarpus marsupium	Leguminoceae	Stem wood	Marsupsin, pterosupin, pterostilbene	Insulinomematic activity	Antidiabetic	[79]
Swertia chirata	Gentinaceae	Whole plant	Amarogentin, swerchirin, chirantin	Stimulates insulin release from islets	Antihyperglycemic agent	[80]
Syzygium aromaticum	Myrtaceae	Flower buds	Eugenol, Caryophylline	Insulin secretion, carbohydrate digestion and absorption	Hypoglycemic agent	[81]
Syzygium cumini	Myrtaceae	Bark, seeds	Jambosine, jambolin, anthocyanins.	α-Glucosidase-inhibiting activity	Anti-hyperglycemic	[82]
Terminalia arjuna	Comberetaceae	Stem bark	Arjunic acid, arjunolic acid, gallic acid.	Stimulates insulin release from islets	Hypoglycemic ativity	[83]
Terminalia chebula	Comberetaceae	fruits	Gallic acid, chebulic acid, chebulanin, ellagic acid, chebulegic acid, chebulinic acid	Secretion of insulin from the β-cells.	Hypoglycemic ativity	[84]
Terminalia belerica	Comberetaceae	fruits	ß- sitosterol, gallic acid, ellagic acid, ethyl gallate, chebulaginic acid.	Insulin secretion, carbohydrate digestion and absorption	Hypoglycemic ativity	[85]
Tinospora cardifolia	Menispermaceae	Leaves and stem	Tinosporine, cordifolide, tinosporide, Barberin.	α-Glucosidase-inhibiting activity, glycolysis	Antidiabetic agent.	[86, 87]
Trigonella foenum graceum	Fabaceae	seeds	Trigonellin, Fenugreekine.	Regeneration of pancreatic β cells, insulin secretion	Antidiabetic activity.	[88, 89]
Vinca rosea	Apocynaceae	Whole plant	Catharanthine, vindoline, vindolinene vinblastine, vincristine	Regeneration of pancreatic β cells, insulin release	Hypoglycemic activity	[90]
Vitis vinifera	Vitaceae	Leaves, stem	E-resveratrol, E-ε-viniferin, anthocyanins.	Insulinonematic activity	Anti-hyperglycemic activity	[91]
Withania somnifera	Solanaceae	Leaves, roots	Withaferin A, withanolides	Insulin release from pancreatic β cells	Hypoglycemic activity	[92]
Zingiber officinalis	Zingiberaceae	rhizomes	Gingirol, shoagol, zingerone.	Increase insulin level & decrease fasting glucose level	Hypoglycemic activity	[93]

Antidiabetic effects of *Gymnema sylvestre*

Various in vivo animal studies and clinical expeiments have repeatedly shown hypoglycemic effects of leaves of G. sylvestre. In a study, the methanol extract of *G. sylvestre* leaf and callus showed pronounced anti-diabetic activity through the regeneration of β-cells [97]. Sugihara Y., et al. reported the antihyperglycemic effect of gymnemic acid IV, isolated from the leaves of *G. sylvestre*. The study reported that gymnemic acid IV decreased blood glucose levels by 13.5 − 60.0% within 6 hours of administration in comparison with glibenclamide, also increased plasma insulin levels was observed in STZ-diabetic mice at a concentration of 13.4 mg/kg due to gymnemic acid IV [66].

Momordica charantia

Momordica charantia also known as karela, the bitter guard is a flowering wine, belonging to family Cucurbitaceae. The herb is commonly used by Ayurvedic and other traditional systems of medicine as an anti-diabetic agent. The plant is widely cultivated in Asia, India, East Africa, and South America [72].

Phytochemistry of *M. charantia*

Bitter melon is rich in constituents such as glycosides, saponins, alkaloids, reducing sugars, resins, phenolic constituents, fixed oil, and free acids [98]. The main phytoconstituents present in *M. charantia* are charantine, charine, momordicin, momordin, cucurbitins, cucurbitacins momorcharins, etc. [72]. Most of the anti-diabetic potential of *M. charantia* is ascribed to Charantin. The hypoglycemic activity of the compound is similar to that of insulin [99].

Mechanism of action

M. charantia exerts its hypoglycemic effects via multiple mechanisms. The possible mechanism of the hypoglycemic action of *M. charantia* is mainly due to insulin secretion and glycogen synthesis [71, 72]. Some studies indicate that bitter melon may stimulate glucose utilization by peripheral and skeletal muscle, inhibit intestinal glucose uptake, and increase hepatic glycogen synthesis [72]. Hsin-Yi Lo et al., reported that seed extract of *M. charantia* regulates glucose metabolism mainly via the insulin signaling pathway [100]. In an experimental study using cell-based screening assay Hsueh-ling Cheng identified and reported that triterpenoids are the potential hypoglycaemic agents responsible for anti-diabetic action of the plant, also the underlying mechanism to bring about the action was attributed to AMP-activated protein kinase [101]; Fig. 2.

Antidiabetic effects of *M. charantia*

Experimental studies have confirmed the hypoglycaemic effect of *M. charantia* on various animal models. A study demonstrated the dose-dependent hypoglycaemic activity exhibited by methanolic fruit extract of *M. charantia* in alloxan-induced diabetic rats [102]. Mahmoud MF et al., studied the

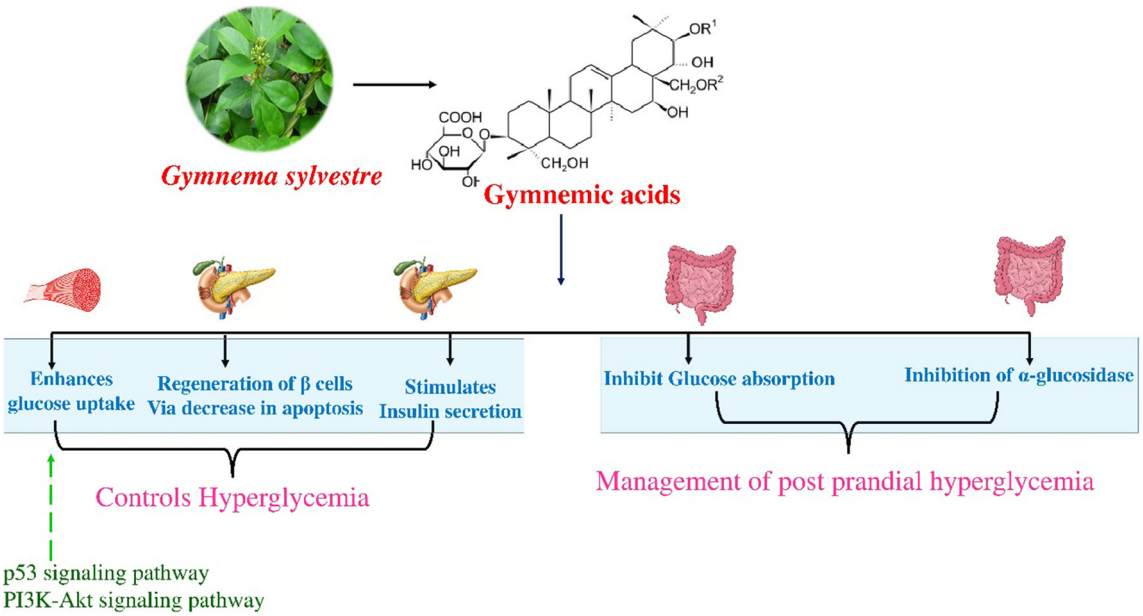

Fig. 1 Schematic representation of Probable molecular mechanism for anti-diabetic effect of *G. sylvestre*

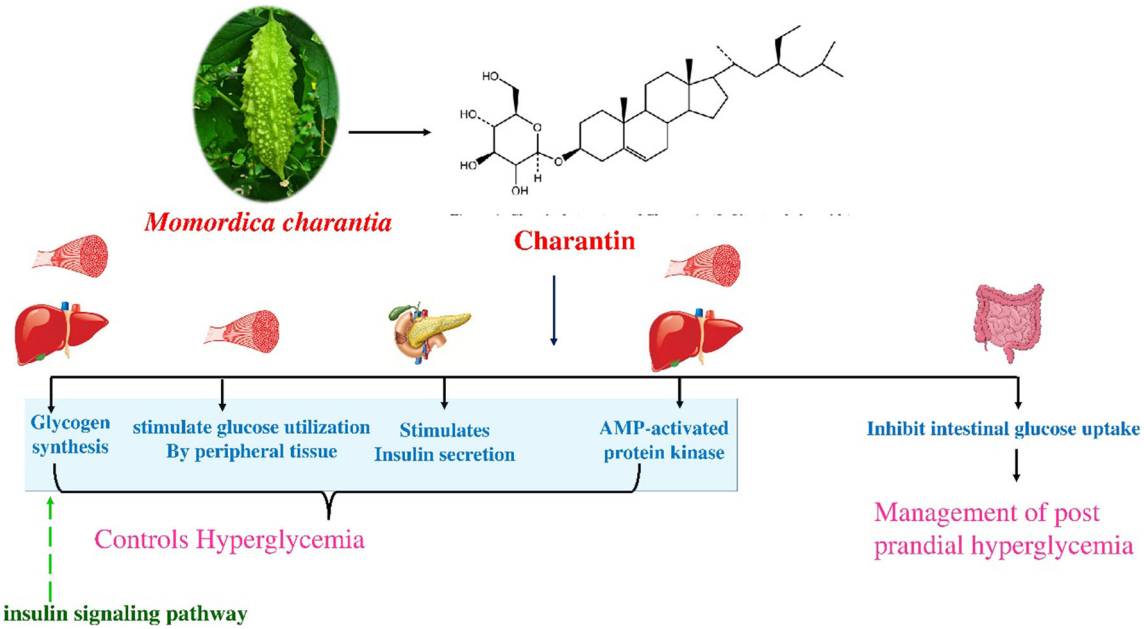

Fig. 2 Schematic representation of Probable molecular mechanism for anti-diabetic effect of *M. charantia*

antidiabetic activity of *M. charantia* fruit juice in streptozotocin-induced diabetic rats [103]. In an investigational study Joo-Hui Han et al., isolated four new cucurbitane-type triterpenoids (C1-C4) from the ethanol extract of *M. charantia* and investigated whether the compounds affect insulin sensitivity both in vitro and in vivo models. The results reported significant decreases in blood glucose level and enhanced glycogen storage by compound C2 in STZ-injected mice [104].

Trigonella foenum graceum

Trigonella foenum graceum also known as fenugreek is used primarily as an alternative therapy for diabetes. A member of the Fabaceae family, the plant cultivated in India and North African countries. The herb has a long history of usage as a potent anti-diabetic agent in Ayurvedic and folklore medicine [94, 105].

Phytochemistry of *T. foenum graceum*

The phytochemical studies have largely been focused on seeds. The main chemical constituents of seeds are alkaloids approximately 36%, steroidal saponins, mucilage, fibers [105]. Among the alkaloid content of fenugreek seed, Trigonelline is major phytoconstituents which is responsible for most of the activity of the herb. The mucilage (25–30%) is mostly a galactomannan. Steroidal saponins such as diosgenin and yamogenin constitute about 0.1–2.2%. Fenugreekine a sapogenin peptide ester is also present in the seeds. The free amino acids in the seeds are present as 4-hydroxyisoleucine, which is reported to have directly stimulated insulin [94, 106].

Mechanism of action

The mode of action of the herb is through Regeneration of pancreatic β cells and insulin secretion [88, 89]. A study reported the inhibitory role of Trigonelline on the activity of glycogen synthase kinase isoforms in the regulation of glycogen metabolism, to bring about the hypoglycaemic action [106]. Trigonelline has also been found to enhance glucose and lipid hemostasis via the improvement of the insulin signaling pathway [107]. Another possible mode of action of *T. foenum graecum*, are the Inhibition of glucose uptake, GLUT-4 translocation, and improved insulin resistance [108, 109]; Fig. 3.

Antidiabetic effects of *T. foenum graecum*

The pharmacological studies have proven the anti-diabetic potential of various extracts of *T. foenum graecum.* In a study, the ethanolic extract of fenugreek seeds was investigated for anti-diabetic action on streptozotocin-induced diabetic rats. The results demonstrated a significant decrease in serum glucose, total cholesterol, triacylglycerol, while an increase in serum insulin in diabetic rats was observed [110]. Shah et al., studied the hypoglycaemic effect of Trigonelline in alloxan-induced diabetic mice. They reported a reduction in blood glucose level and identified the presence of islet cells in the pancreatic duct suggesting its beneficial effect on β cells [111]. Multiple studies on seed extracts, raw powder, and active constituents by investigators have demonstrated the hypoglycaemic action of the herb, confirming its use as a potent herbal remedy for the management of diabetes.

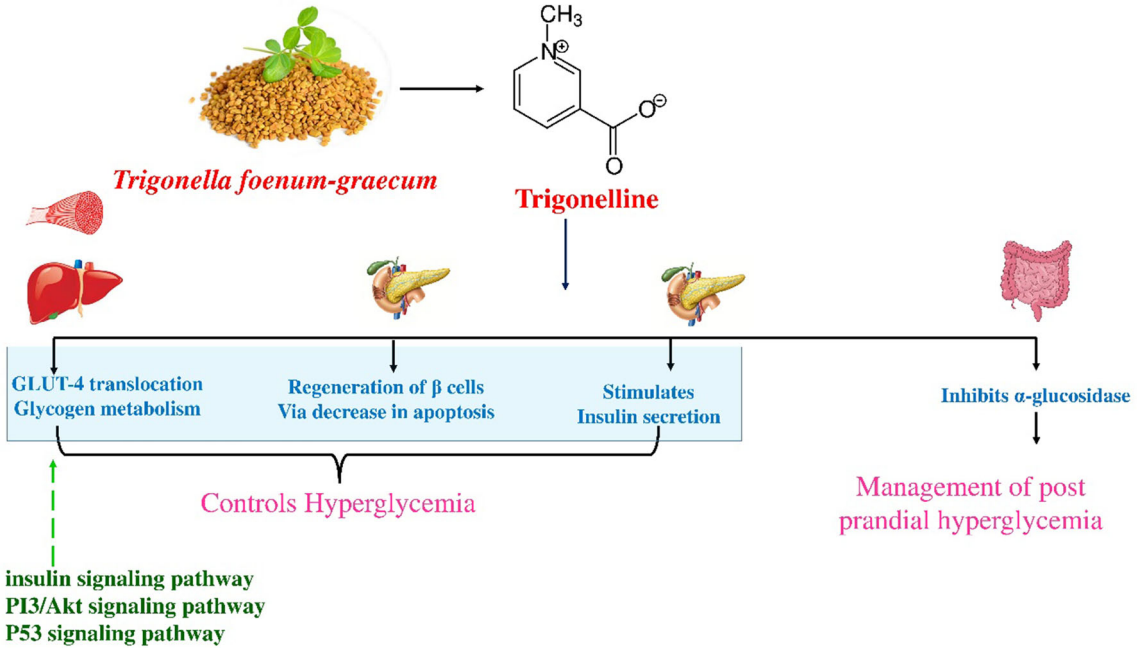

Fig. 3 Schematic representation of Probable molecular mechanism for anti-diabetic effect of *T. foenum-graecum*

Tinospora cordifolia

Tinospora cordifolia (Willd.) Miers, belonging to family Menispermaceae, is a potent herb used to combat diabetes. The herb reported to possess anti-diabetic activity in Ayurvedic literature and is present in many Ayurvedic formulations. The herb is commonly known as Guduchi and is indigenous to the tropical areas of India, Myanmar, and Sri Lanka [94].

Phytochemistry of *T. cordifolia*

T. cordifolia consists of a variety of phytoconstituents belonging to different classes such as alkaloids, glycosides, steroids diterpenoid, sesquiterpenoid, phenolics, proteins, etc. The major active constituents responsible for the anti-diabetic effect belongs to a class of alkaloids; these consist of Berberine, Palmatine, Tembetarine, Magnoflorine, Tinosporin. The other constituents present are Tinocordiside, Tinocordifolioside, Cordioside, Cordifolioside, Tinosporon, Tinosporides, etc. [94, 112].

Mechanism of Action

T. cordifolia is reported to act by a different mechanism of action. The possible mechanism to bring about hypoglycemic action is due to inhibition of α-glucosidase activity and glycolysis. In a study Chougale et al. reported the inhibitory effect of *T. cordifolia* on the α-glucosidase enzyme [86]. Joladarashi et al., proved the Glucose uptake-stimulatory effect of stem extracts of *T. cordifolia* by conducting experiments with Ehrlich ascites tumor cell model. The study reported the ameliorotary effect of *T. cordifolia* on GLUT 1 and GLUT 3 transporters involved in basal glucose uptake; suggesting it as a possible mechanism to exert the hypoglycemic effect [113]. Another study reported the insulin-releasing, insulin-sensitizing, and inhibition of gluconeogenesis as the possible mechanism exhibited by an alkaloidal fraction of *T. cordifolia* [114]; Fig. 4.

Antidiabetic effects of *T. cordifolia*

The stem has been the maximum investigated part of the plant for its anti-diabetic activity. It has been reported that methanol extract of *T. cordifolia* significantly reduces the fasting blood glucose levels in streptozotocin-induced diabetic rats. Improvement in the insulin and C-peptide levels were also reported which indicated the regeneration of β cells in the pancreas [115]. Manikkam et al. isolated a polysaccharide from methanolic extract of *T. cordifolia* stem and demonstrated the β-cell regenerative property of the isolated polysaccharide in streptozotocin-induced diabetic rats suggesting its usage as a potent hypoglycemic agent [116].

Curcuma longa

Curcuma longa Linn, belonging to family Zingiberaceae, is reported as a potent herb in Ayurveda system of medicine to combat diabetes. It is commonly known as Turmeric, Haldi,

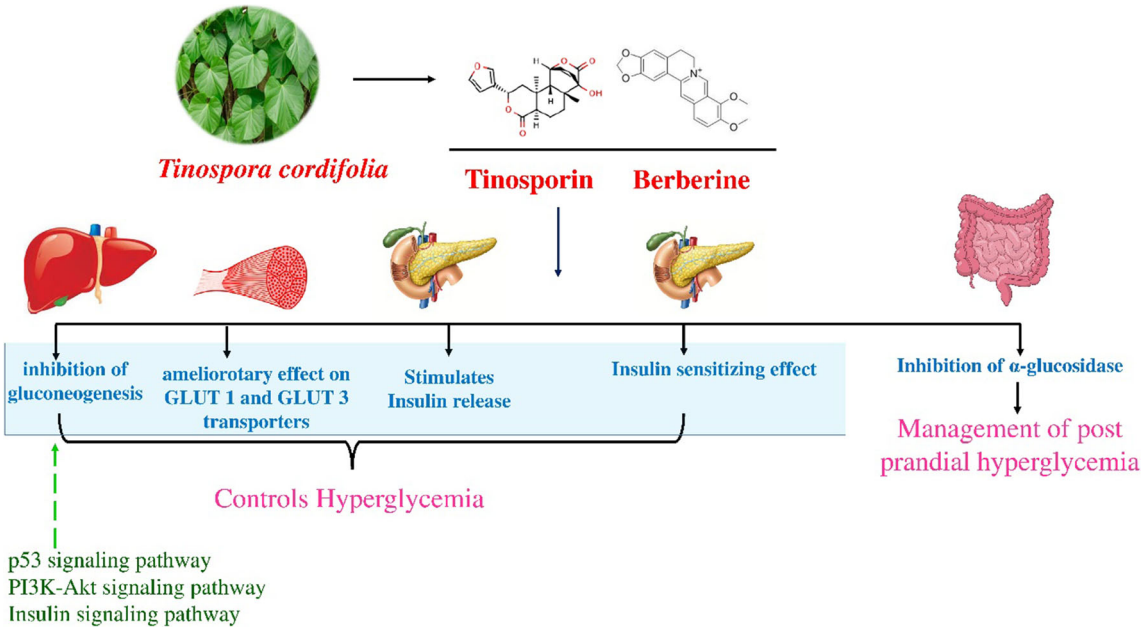

Fig. 4 Schematic representation of Probable molecular mechanism for anti-diabetic effect of *T. cordifolia*

Haridra, etc. The herb is native to India and is widely cultivated particularly in West Bengal, Tamil Nadu, and Maharashtra [94].

Phytochemistry of *C. longa*

The rhizomes of *C. longa* consist of a large number of phenolic compounds. Curcuminoids are the major active constituents present in the rhizomes. Curcuminoids are the mixture of three related compounds namely Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin among this curcumin constitute about 60% of total curcuminoids. Curcumin is a major active principle responsible for most of the biological activity of *C. longa* [94].

Mechanism of action

C. longa is known to exert the hypoglycemic action via different mechanisms, of which the most common being the inhibition of α-glucosidase and α-amylase enzyme [52, 117,

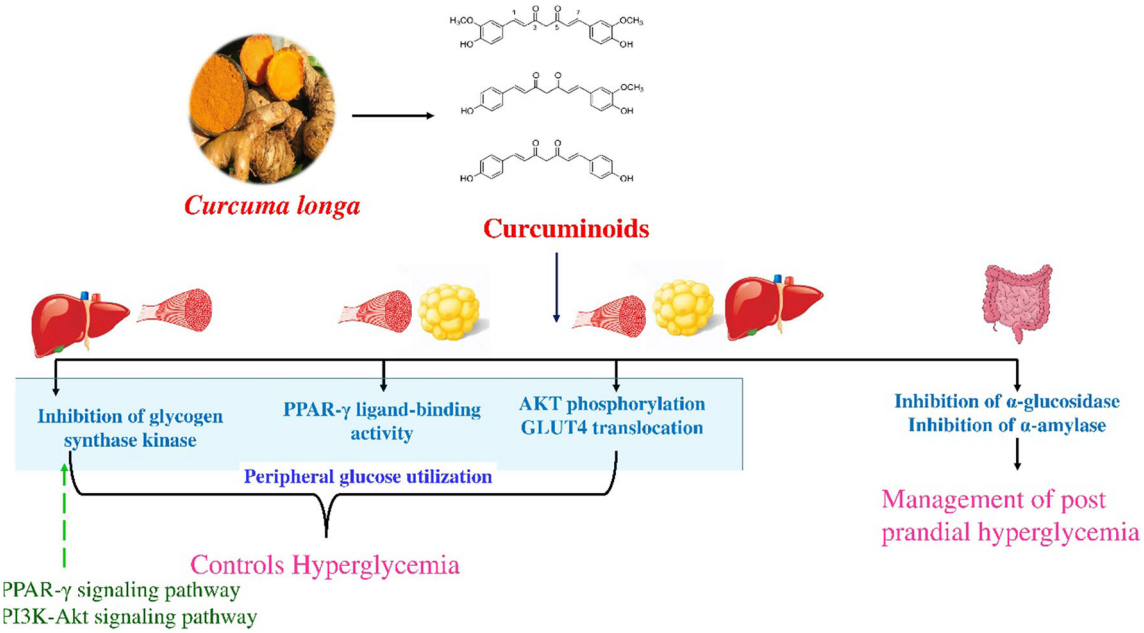

Fig. 5 Schematic representation of Probable molecular mechanism for anti-diabetic effect of *C. longa*

Table 2 Clinical studies on Anti-diabetic efficacy of selected five plants

Author (year)	Plant name	Study	Duration	Dose	Number (Cases/ Control)	Outcome	References
Gaytan Martinez L. et al., (2020)	*G. sylvestre*	RCT Double-blind	12 weeks	600 mg/day (GS capsule)	15/15	↓ 2-h OGTT ($p = 0.003$) and A1C ($p = 0.025$), ↑ insulin sensitivity	[122]
Al-Romaiyan A. et al., (2010)	*G. sylvestre*	Cohort study	2 months	1 gm/day (Novel GS extract)	11	↓ blood glucose ↑plasma insulin and C-peptide levels	[123]
Nanda Kumar S. et al., (2010)	*G. sylvestre*	quasi-experimental design	3 months	500 mg/day (GS capsule)	39/19	↓ blood glucose (fasting and post-prandial), and glycated hemoglobin	[124]
Trakoon-osot W. et al., (2013)	*M. charantia*	RCT Double-blind	16 weeks	6 g/day of MC dried-fruit pulp	19/19	↓ A1C from baseline ($p = 0.042$, ↓ of total advanced glycation end products (AGEs) in serum ($p = 0.028$)	[125]
Fuangchan A. et al., (2011)	*M. charantia*	Multicentric double-blind RCT	4 weeks	G1-500 mg/day, G2-1000 mg/day, G3-2000 mg/day, dry fruit pulp (G4-1000 mg/day metformin)	G1 = 33 G2 = 32 G3 = 31 G4 = 33	↓ fructosamine levels in G3 and G4.	[126]
Lim ST. et al., (2010)	*M. charantia*	RCT double-blind		G1- 60 mg/kg/day G2-80 mg/kg/day G3-100 mg/kg/day G4 = Placebo	G1 = 10 G2 = 10 G3 = 10 G4 = 10	G3 showed a more rapid (15 minutes) stimulation of insulin secretion than placebo	[127]
Najdi RA. et al. (2019)	*T. foenum graecum*	RCT	12 weeks	2 gm/day TGF	6/6	↑ fasting insulin level ($P = 0.04$).	[128]
Verma N. et al., (2016)	*T. foenum graecum*	Multicentric double-blind RCT	3 month	1000 mg/day Fenfuro (TGF seed extract) capsule	154	↓ fasting plasma, post-prandial blood sugar levels and HbA1c levels. ↑ fasting and post-prandial C-peptide levels.	[129]
Kumar V. et al., (2015)	*T. cordifolia*	RCT	15 days	50 mg/ kg body weight/ day TC stem powder	90	↓ fasting blood sugar	[130]
Roy K. (2015)	*T. cordifolia*	RCT	2 Months	500 mg/day encapsulated stem of TC	29/30	↓ HbA1c levels	[131]
Rahimi HR. et al., (2016)	*C. longa*	RCT Double-blind	3 months	80 mg/day Nano-curcumin (as nano-micelle)	39/41	↓ fasting blood sugar ($p = 0.004$) ↓ HbA1c levels ($p = 0.02$)	[132]
Chuengsamarn S. et al. (2012)	*C. longa*	RCT Double-blind	9 months	1500 mg/day curcuminoids capsule	119/116	↓ fasting plasma glucose and HbA1c levels ($p = < 0.01$), better β cell functions. ↑HOMA-β ($p = < 0.01$)	[133]
Na LX. et al. (2012)	*C. longa*	RCT Double-blind	3 months	300 mg/day curcuminoids	50/50	↓ fasting blood glucose ($p < 0.01$), HbA1c ($p = 0.031$), and insulin resistance index (HOMA-IR) ($p < 0.01$)	[134]

118]. Gutierres et al.., reported that increased levels of AKT phosphorylation and GLUT4 translocation in skeletal muscles could be the possible mechanism responsible for the antidiabetic activity of curcumin [53]. Kuroda et al., reported that the hypoglycemic effect exerted by curcumin, demethoxycurcumin, bisdemethoxycurcumin, and ar-turmerone is mainly attributed to PPAR-γ ligand-binding activity of the compound [119]. In a molecular docking study Yasser et al., reported that the hypoglycemic effects of curcumin may be due to the Inhibition of glycogen synthase kinase 3β [120]; Fig. 5.

Antidiabetic effect of *C. longa*

Various studies have shown the hypoglycemic activity of rhizomes of *C. longa*. Seo et al. investigated the glucose-lowering potential of curcumin in diabetic db/db mice. A significant decrease in blood glucose and HbA1c levels were observed in animals treated with curcumin. A further study reported the improvement in glucose homeostasis, glucose tolerance, and elevated plasm insulin levels by the administration of curcumin [121]. A study reported the suppression of increased blood glucose levels in Genetically Diabetic KK-A^y Mice by ethanolic extract *of C. longa* [119].

Clinical studies

Clinical trials plays an important role in accessing the safety and efficacy of a particular medication in humans. Based on the literature obtained on the clinical efficacy of the selected five herbs, only recent publications from the year 2010 onwards have been identified from the database search. The details of clinical experiments for the investigation of anti-diabetic effects of the five herbs has been summarized in Table 2.

Conclusion

Diabetes mellitus is the most common endocrine disorder marked by persistent hyperglycaemia resulting from impaired insulin production or insulin resistance. Regardless of all the developments in therapeutics, diabetes still remains a major cause of morbidity and mortality in the world. Allopathic therapies available currently for the treatment of diabetes have a number of serious side effects; consequently, there is a need for investigation of more effective and safer hypoglycaemic agents. Traditional medicine and ethno-botany have an ever-emerging role to play in the treatment and management of diabetes mellitus.

The present review has highlighted the usefulness of the herbal source for the treatment and management of diabetes mellitus. Around 50 herbs known for their usefulness in diabetes have been reviewed and a possible mechanism of the action exerted by them to bring about the anti-diabetic action has been highlighted. Among them, light has been shed upon 5 most potent anti-diabetic herbs with respect to their phytochemistry, underlying mechanism of action, and anti-diabetic effect exerted by them.

From the evidences gathered from literature it is noticeable that the herbs acts by various mechanism to bring about the anti-diabetic effect. As evident from the literature a single herbs displays multiple mechanism of action for example Regeneration of pancreatic β cells, inhibition α-glucosidase enzyme, insulin secretion, PPAR-γ ligand binding activity, etc. this may be due to the presence of a variety of phytoconstituents in a herb. This can in turn bring about the synergistic effects leading to reduction in hyperglycaemic action. As described in the review, most of the reported mechanism of actions exerted by the herbs have been described. In addition to that, more information on *G. sylvestre, M. charantia, T. foenum graecum, T. cordifolia and C. longa* has been provided owing to their extensive utilization by herbal industries for development of anti-diabetic products. By looking at the diverse phytoconstituents, their mechanism of action, and clinical evidences it is clear that these herbs possess anti-diabetic effect.

Based on this comprehensive overview we can conclude that herbal medicines can play a pivotal role in the management and treatment of diabetes with fewer side effects. More investigations mainly focusing on the isolation of phytocompounds from these herbs can lead to the discovery of newer anti-diabetic agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Title of Paper: Quality by Design assisted RP-HPLC method development and validation for the quatification of gymnemagenin in *Gymnema sylvestre*

Paper code: PP10

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