Research Square

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Fusion, Micronization and Bio-activated (FMB) extract of *Gymnema sylvestre* for higher antidiabetic and antioxidant effects compared to its conventional standardized extract

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Research Article

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Abstract

Despite considerable studies, diabetes continues to remain a challenge for its prevention and management with an increasing rate of morbidity and mortality. One of the popular herbal extracts - Gymnema sylvestre has been extensively studied for its anti-diabetic potential. The present study aimed to explore the *in vitro* antidiabetic and antioxidant effects of *Gymnema sylvetsre* (Gudmar; GD) and also reveal its drug-likeness, toxicity and action mechanism by utilizing in silico molecular docking and network pharmacology, respectively. The elemental analysis confirmed that FMB technology successfully replenishes iron, zinc and calcium in the conventional extract which is devoid of these essential trace elements. GD extract-FMB (IC_{50} = 131.90 ± 0.44) are more effective as compared to conventional GD extract in the case of α-amylase (IC_{50} = 118.23 ± 0.42; IC_{50} = 109.52 ± 0.34) and α-glucosidase (IC_{50} = 109.52 ± 0.34), respectively while a similar effect was observed in DPPH-free radical scavenging (IC_{50} = 129.2 ± 0.98) of GD extract-FMB as compared to GD extract (IC_{50} = 176.6 ± 0.51). Network pharmacology revealed that key target proteins that are strongly linked to iron, zinc and calcium were strongly involved in glucose metabolism, oxo-inflammatory responses and insulin-related pathways. In addition, in silico molecular docking results of gymnemic acid against α-amylase and α-glucosidase revealed strong biological bindings. Conclusively, based on the finding we can conclude that FMB technique replenish the ETEs in the conventional extract. Also FMB processing is beneficial and provides a innovative solution for the herbal pharmaceutical industry to develop better efficacious extracts or herbal formulations.

1. Introduction

Diabetes, often known as diabetes mellitus, is a deadly state characterized by high blood sugar levels in the circulatory system [1]. Worldwide, 536.6 million individuals were anticipated to have diabetes in 2021, and that number is projected to increase to 783.2 million by 2045. Additionally, about 541 million people who have impaired glucose tolerance are at a high risk of getting diabetes, and it was predicted that the cost of healthcare worldwide will reach USD 966 billion in 2021 [2]. Diabetes requires urgently appropriate therapy due to its high prevalence, varied pathophysiology, and consequences. Inhibiting the enzymes α-amylase and α-glucosidase is one way to manage diabetes by preventing the body from absorbing glucose [3]. The starch is randomly broken down into simple saccharides by salivary and pancreatic α-amylase, resulting in smaller molecules of glucose that are taken into the bloodstream. The α-amylase inhibitors either stop starch from being broken down into simple sugars or slow down the digestive system's absorption of sugar. Animal tissues include an exo-type carbohydrate called α-glucosidase, which catalyses the release of glucose from the substrate's non-reducing end. It is a membrane-bound enzyme that helps the small intestine's brush border membrane break down oligosaccharides into monosaccharides that may be absorbed to help with glucose absorption [4]

According to the WHO, more than 80% of people utilise herbal medicine to halt the spread of numerous diseases and ailments in underdeveloped nations. Numerous pharmaceutical businesses focus on developing herbal medicines that slow the growth of diabetes and related illnesses despite the availability

of standard antidiabetic medications [5, 6]. Plants have a lot of untapped potential as a source for developing novel medications. One of these is *Gymnema sylvestre*. *G. sylvestre* includes a huge number of phytoconstituents, such as gymnemic acid, gymenemagenin, caffeic acid, gallic acid, quercetin, kaempferol etc., which are the substances responsible for antioxidant, liver protection, insulin secretion, cardioprotection, intestinal health, cancer prevention, and many other functions [7, 8].

Phytoconstituents extracted in the present day processes of making herbal extracts, omits or eliminates the ETEs that can lead to the lower pharmacological potential of the extract. In the present study, we have successfully implemented a process technology of FMB (fusion (F), micronization (M), and bioligation (B) [9] in conventional extract of *G. sylvestre* to increase its therapeutic potency. This method is simple and can be used to create extracts and formulations that are ready to use in terms of their phytochemical contents and ETEs, as well as to reach higher pharmacological potentials. Prior experimental, research showed that the use of phytoconstituents and essential trace elements (ETEs) in the management of diabetes was linked to their capacity to impede the digestion of carbohydrates by blocking the enzymes α-amylase and α-glucosidase. Therefore, combining phytoconstituents and ETEs may have great potential as enzyme inhibitors in the treatment of diseases including diabetes [10]. There are currently no published studies on the function and mode of action of phytoconstituents in conjunction with ETEs for the treatment of diabetes.

The pharmacological impact of a medicine can be explained or predicted using computer-aided approaches, which is intriguing for the quick screening of bioactive components in a mixture of plant-based medicines [11]. Additionally, computational methods of pharmacological screening of lead compounds decrease the expense of the experiment, the amount of time spent in discovery, and most importantly, they minimise the number of investments and frills made at any stage of drug development of bioactive leads from causing a significant loss to the organization [11, 12]. The comprehensive analysis of pharmaceuticals and drug targets via network pharmacology and in silico molecular docking, however, may provide us with a profound understanding of how drugs work. It is now widely recognised as a useful instrument for systematic evaluation and for demonstrating the logic of medications. Keeping this in view, the present study aims to explore the enzyme inhibitory potential of *G. sylvestre* and also reveal the action mechanism by utilizing in silico molecular docking and network pharmacology, respectively.

2. Materials And Methods

Conventional *G. sylvestre* extract (GD extract) was purchased from an established herbal extract manufacturer and its FMB extract (GD extract-FMB) was prepared in-house. After the process, *in vitro* biological investigations were carried out to find its biological application.

Step 1

Separation of ETEs (iron, zinc and calcium, *etc.*) from Gudmar raw material.

Step 2

Replenish the ETEs into conventional extract to develop novel herbal extract as follows

I. Fusion: Involves replenishment of conventional herbal extract with the ETE lost during the process of extraction.

II. Micro-fixation: Micro-fixation of conventional extract and ETEs is done through an incremental and continuous process of micronization.

III. Bio-activation: Both fusion and micro-fixation help bio-activate the extract through formaltion of complex between ETEs, and bioactive phytoconstituents present in the conventional extract.

2.1. Determination of ETE

The operational conditions for determining ETE in the samples were carried out in line with the described procedure [13]. Each sample was digested in a microwave digester with 0.5 g in 5 ml of ultrapure, metal-free nitric acid. After that, using an inductively coupled plasma and an optical emission spectroscope, the contents were diluted to a volume of 12.5 ml with double-distilled water (ICP-OES). The digested sample was aspirated into an ICP-OES to quantify ETEs such as iron, zinc, and calcium. The experiment was carried out three times, and the mean and standard deviation of the outcomes were presented.

2.2. Determination of total phenol and flavonoids

The Folin Ciocalteu (FC) method was used to determine the total phenol content with little modification. In a nutshell, a stock solution of extract in methanol (5 mg/mL) was made. To a 500 mL stock solution, 2.5 mL FC (1:10, v/v) was added. After mixing 2.5 mL of a 7.5% sodium bicarbonate solution was mixed and left for 30 minutes while being occasionally shaken. The absorbance at 765 nm was measured with a spectrophotometer. To calculate the total phenolic content, a calibration curve was drawn between absorbance and standard concentrations. The result was given as mg gallic acid equivalent per gramme of extract (mg GAE/gm extract). A modified aluminium chloride technique was used to measure the total flavonoid content. The extract was diluted in methanol to make a stock solution with a 5 mg/mL concentration (500 L of stock solution to 1.5 mL methanol). After waiting for five minutes, 0.1 mL of aluminium chloride (10%), 0.1 mL of sodium acetate (1M), and 2.8 mL of water were added. After incubating for 30 minutes, a spectrophotometer was used to detect the absorbance at 415 nm. The result was represented as milligrammes of quercetin equivalent per gramme of extract [14].

2.3. Antioxidant activities

According to the established protocol, the capacity of GD extract-FMB and GD extract to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) was examined. The samples were combined with a 0.1 mM DPPH solution and incubated at 37°C in the dark for 30 min. The scavenging potential was then calculated by measuring the absorbance at 517 nm. Graph Pad Prism was used to determine the IC_{50} values for the reference substance, quercetin [15].

2.4. Determination of α-amylase and α-glucosidase inhibition potential

The capacity to inhibit α-amylase and α-glucosidase was examined using different concentrations of GD extract-FMB and GD extract (33–500 g/mL). For α-amylase, 1.0 mL of extracts (each concentration), 1.0 mL of amylase enzyme, and 1.0 mL of starch solution were mixed and maintained at 37°C for 30 minutes. Furthermore, the inhibitory action was evaluated using a glucose reagent in 100 µL of supernatant. For α-glucosidase, 120 LµL of different extract concentrations (33–500 g/mL) and 20 µL of glucosidase were added to phosphate buffer saline (PBS) and incubated at 37°C for 15 min. 20 µL of 5 mM p-nitrophenyl-D-glucopyranoside (made in PBS) were mixed to begin the reaction, which was then left to stand for 15 minutes. The reaction was stopped by adding 80 µL of sodium carbonate that had been dissolved in PBS before measuring the absorbance at 405 nm. Acarbose was utilised as a positive control, and IC_{50} values were computed using Graph pad prism [3].

2.5. Target genes related to selected compounds and diabetes mellitus

Swiss Target Prediction (STP) in "Homo Sapiens" mode was used to choose target genes based on the compound SMILES. Diabetes-related genes were discovered by DisGeNET.

2.6. Protein-protein and ETEs interaction (PPI) network

In order to create the PPI to describe the interaction between target proteins and ETEs, the target proteins of related *G. sylvestre* compounds were uploaded to the Cytoscape software. The correctness of this analysis was therefore ensured in the current investigation by the use of high-confidence data. We've also built a network connecting the disease and key genes involved in the pathophysiology of diabetes and disorders that are related to it.

2.7. In silico molecular docking

2.8. Target protein sequence retrieval and preparation of bioactive leads

From the Research Collaborators for Structural Bioinformatics Protein Data Bank (RCSB PDB) database, the α-amylase and α-glucosidase proteins were downloaded in FASTA format. Guymnemic acid, one of the ligands, was retrieved from PubChem as a 3D structure SDF file and optimised using Ligands Input in AD 4.2.

2.9. Preparation of targeted enzyme and molecular docking

The target enzyme was constructed by adding all polar hydrogen atoms and removing the water molecule and other heteroatoms using AutoDock. All of the rotatable bonds were set to be rotatable and Gasteiger charges were added to the ligands. Using Autodock 4.2, the improved ligand molecules were

docked with improved diabetes receptors. PyMOL, a programme for visualising molecular graphics, was used to assess the docking results. The optimal conformation was chosen from the most densely populated cluster with the lowest binding energy following the conclusion of docking searches.

3. Results And Discussion

3.1. Elemental analysis

Results presented in Table 1 showed the levels of ETEs (Iron, Zinc and Calcium) concentration in GD extract-FMB and GD extract, respectively. From the result, it is ascertained that ETEs were successfully replenished into a conventional extract, *i.e.*, GD extract-FMB.

Table 1
Comparative content of ETEs analyzed by ICP-OES (n = 3)

(ETEs)	GD extract-FMB (Units in ppm)	GD extract (Units in ppm)
Iron	93 ± 4.01	12.00 ± 0.61
Zinc	36.89 ± 3.22	18.69 ± 0.97
Calcium	3100.00 ± 43.65	139.66 ± 2.04

Iron plays a direct and causal involvement in the aetiology of diabetes, and two significant mediators of this role are insulin resistance and beta-cell failure. Iron is used by the majority of tissues involved in fuel homeostasis to control metabolism, with the adipocyte being crucial in iron sensing [16]. Previous studies reported that concurrent administration of ETE including iron reduces oxidative damage and increases antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase [17].

Zinc plays a significant role in antioxidant defence in type 2 diabetic patients by functioning as a cofactor of the superoxide dismutase enzyme, regulating glutathione metabolism and metallothionein expression, competing with iron and copper in the cell membrane, and inhibiting the nicotinamide adenine dinucleotide phosphate-oxidase enzyme. Zinc reduces persistent hyperglycemia, which reduces oxidative stress in these people. It fact it promote insulin receptor phosphorylation by enhancing glucose transport into cells [18, 19]. A previous study reported that the Zn-coupling extract of *Catharanthus roseus* showed better α-amylase inhibitory activity as compared with a standard drug (Acarbose) [20].

Calcium is essential for insulin-mediated intracellular activities in tissues that respond to insulin, such as skeletal muscle and adipose tissue. However, effective insulin-mediated activities only need a small number of calcium ions for cellular performance [21]. Calcium decreases reactive oxygen species and boosted antioxidant enzyme activity in the cells. [22]. Additionally, a previous study found that patients with type 2 diabetes have abnormalities in skeletal muscle, adipocytes, and liver that are associated with impaired cellular calcium homeostasis. Calcium also aids in reducing de novo lipogenesis by increasing insulin activity and reducing fat accumulation [23].

3.2. Estimation of total phenolic and flavonoid content

The calibration curves of gallic acid (r^2 = 0.9954) and quercetin (r^2 = 0.9929) were used to calculate total phenolic and flavonoid content. The total phenolic and flavonoid content per gram of gallic acid and quercetin was found to be 16.34 ± 0.46 mg/g and 2.18 ± 0.74 mg equivalents, respectively. Our findings showed that *G. sylvestre* is high in phenolics and flavonoids.

Phytoconstituents especially polyphenols which are abundant in plants may have a beneficial effect on hyperglycemia by inhibiting the activity of carbohydrate-digesting enzymes (α-amylase and α-glucosidase), stimulating insulin secretion from pancreatic beta-cells, and decreasing blood glucose levels [24]. Moreover, the cited studies demonstrated that phytoconstituents protected beta-cells and their integrity by modulating hyperglycemia and oxidative stress [25]. The identification of gallic acid and quercetin in the present work strongly supported the pharmacological significance of *G. sylvestre* for its antioxidant and antidiabetic potential.

3.3. Free radical scavenging assay

DPPH free radicals scavenging ability of extract was calculated as percentage inhibition. In the present study, DPPH screening of GD extract-FMB and GD extract had clearly shown the dose-dependent antioxidant activity at the different concentrations tested (33–500 µg/mL). At maximum tested concentration, the inhibition potential of GD extract-FMB, GD extract and quercetin was 84.29 ± 1.06, 79.21 ± 1.13 and 95.29 ± 0.48%, respectively. The obtained results revealed antioxidant inhibition by the GD extract-FMB is more effective and similar to the reference compound as compared to the GD extract. The overall data was presented in **Table 2**.

Concentration (µg/mL)	DPPH activity		
	Quercetin	GD extract-FMB	GD extract
33	33.87 ± 0.16	18.44 ± 0.30	13.67 ± 0.34
66	56.43 ± 0.37	31.57 ± 0.54	26.87 ± 0.68
120	69.08 ± 0.72	45.87 ± 0.48	39.01 ± 0.19
250	81.34 ± 0.28	69.54 ± 0.96	56.07 ± 0.66
500	95.29 ± 0.48	84.29 ± 1.06	79.21 ± 1.13
IC_{50}	**57.00 ± 0.62**	**129.20 ± 0.98**	**176.60 ± 0.51**

The DPPH evaluation is a simple and reliable procedure to assess the antioxidant properties of herbal products. *G. sylvestre* contains a lot of phytoconstituents, which slow down the oxidation of organic matter by adding a hydrogen atom to the chain-carrying ROO* radicals [26]. Through this mechanism, phytoconstituents inhibit the formation of free radicals and play a pivotal role in reactive oxygen species metabolism in the biological system.

3.4. In vitro α-amylase and α-glucosidase inhibition activity

FMB extract and conventional extract of *G. sylvestre* were tested against the carbohydrates digesting enzyme α-amylase and α-glucosidase and their inhibitory potential was found in a concentration-dependent manner, implying its potential role in diabetes management (33–500 µg/mL). In the case of α-amylase, the IC_{50} value of GD extract-FMB and GD extract was found 118.23 ± 0.42 and 153.45 ± 0.52μg/mL, respectively whereas in the case of α-glucosidase IC_{50} value was found to 109.52 ± 0.34 and 138.72 ± 0.48µg/mL, respectively. The overall data were presented in **Table 3**.

Concentration (µg/mL)	Acarbose	α-amylase		α-glucosidase	
		GD extract-FMB	GD extract	GD extract-FMB	GD extract
33	21.16 ± 0.14	16.26 ± 0.56	12.56 ± 0.67	17.22 ± 0.64	14.62 ± 0.47
66	35.56 ± 0.47	28.43 ± 0.67	25.56 ± 0.86	34.38 ± 0.93	29.88 ± 0.38
120	48.29 ± 0.36	52.69 ± 0.48	41.42 ± 0.48	51.69 ± 0.46	46.48 ± 0.48
250	76.36 ± 0.70	71.80 ± 0.62	65.36 ± 0.94	75.73 ± 0.49	63.78 ± 0.39
500	97.21 ± 0.92	91.82 ± 1.02	83.28 ± 0.47	90.78 ± 0.38	87.54 ± 0.84
IC_{50}	**105.20 ± 0.88**	**118.23 ± 0.42**	**153.45 ± 0.52**	**109.52 ± 0.34**	**138.72 ± 0.48**

One treatment strategy for diabetes management is to hinder/block the absorption of glucose in the gastrointestinal tract and also maintain glucose metabolism in the liver by inhibiting the carbohydrate-metabolizing enzymes α-amylase and α-glucosidase [27, 28]. Likewise, in the current study, α-amylase and α-glucosidase inhibitory activity of GD extract-FMB and GD extract is most likely to be due to number of phytoconstituents including gymnemic acid, gymnemagenin, caffeic acid, gallic acid, quercetin, kaempferol, etc. In the case of both the carbohydrate-digesting enzymes such as amylase and glucosidase, it was found that GD extract-FMB showed a more pronounced effect as compared to standardized extract i.e. GD extract. Furthermore, there has been substantial scientific studies evidence suggesting that inhibition of α-amylase and α-glucosidase by phytoconstituents and ETEs makes it possible to ameliorate hyperglycemia to combat the occurrence of diabetes [29].

3.5. Gene disease association network and PPI network with essential trace elements (ETEs)

In this study, a target-disease interaction network was created using NetworkAnalyst to better understand the relationship between target proteins and disease association (Fig. 2). A total of 44 proteins were screened to describe the pathways involving diabetes and its associated disease. In addition, we have also created an interaction network between proteins associated with diabetes and ETEs (iron, zinc, calcium) using Cytoscape to better understand the relationship between target proteins and ETEs (Fig. 3).

These target proteins were found to be primarily involved in diabetes mellitus, insulin resistance, impaired glucose intolerance, hyperglycemia, hyperinsulinemia, and obesity. According to network analysis, multiple target proteins and ETEs exist in one pathway, and the same target protein and ETEs exist in multiple pathways. In essence, the interaction between one target protein and multiple pathways with ETEs is more significant than the interaction between multiple target proteins and a single pathway with no ETEs. Because we all know that ETEs play a cofactor at the site of receptors that may enhance the bioactivity of ligands (bioactive phytoconstituents). These findings imply that the potent pharmacological components of GD extract-FMB may act on various proteins and signalling pathways to alleviate the levels of insulin, glucose, inflammation, and oxidative stress associated with diabetes and other related diseases [30, 31].

Moreover, biopotential genes (IRS1, TNF-α, IL6, MAPK3, DPP4, LEPR, PPARA and PIK3CG etc.) in the same PPI network were selected in the study to understand the interaction of ETEs of *G. sylvestre* (Fig. 3). The network created in Fig. 2 provided an understanding of the complex network relationships between proteins and disease while Fig. 3 provided the network interaction between proteins and ETEs. The above networks provide multiple target strategies to treat or manage diabetes and associated disease and disorders.

3.6. Molecular docking analysis

For examining the relationship between a protein and its ligand or inhibitor, AutoDock is a widely utilised platform. In order to investigate the interactions between gymnemic acid an active phytoconstituents of *G. sylvestre* and the proteins (α-amylase and α-glucosidase), molecular docking was carried out. The types of chemical bonds that were produced and the binding locations of amino acid residues provided evidence of the interaction between ligand and protein (Table 4). The molecular docking results are presented in Fig. 4. The results of docking studies revealed that gymnemic acid had strong binding with both the enzymes with the lowest energy at 6.88 kcal/mol and 7.74 kcal/mol, respectively that calculated from the docking experiment, and this result agreed with our in vitro results presented above [7].

Table 4
The results of molecular docking of gymnemic acid with the enzymes α-amylase and α-glucosidase.

Compounds	Protein	Binding energy	H-bonds Interactions
Gymnemic acid	α-amylase	-6.88 kcal/mol	Lys200 (A); Glu240 (A)
	α-glucosidase	-7.74 kcal/mol	Lys91 (A); Try307 (A), Glu242(B), Glu48(B), Arg46(B),

Furthermore, the strong interaction of gymnemic acid with α-amylase and α-glucosidase suggested that *G. sylvestre* reduced carbohydrate digestion and absorption. It is also reported that these compounds controlled insulin levels in the pancreatic beta-cells by enhancing the 5' adenosine monophosphate-activated protein kinase pathway [32, 33]. Furthermore, previous findings also suggested that gymnemic

acid may control oxo-inflammations, insulin resistance, and glucose metabolism, which are beneficial for the management of diabetes.

Overall, previous and present evidence reveals that consumption of ETEs and bioactive phytoconstituents exert better pharmacological activity and their collective intake is more beneficial as compared to the solitary application of either phytoconstituents or ETEs. Moreover, the in vitro studies, network pharmacology and in silico molecular studies revealed that FMB extracts are more beneficial and effective as compared to conventional extracts.

4. Conclusion

The prepared GD extract-FMB of *G. sylvestre* significantly inhibited carbohydrate-digesting enzymes α-amylase, and α-glucosidase and excellently scavenge DPPH as compared to its conventional extract, which may be attributed to the successful replenishment of the ETEs into conventional extract through FMB technique. It can be concluded that FMB technique of processing extracts is beneficial and provide a direction for the herbal pharmaceutical industry to develop better therapeutic potential and efficacious extracts or herbal formulations.

Declarations

CONFLICTS OF INTEREST

The authors are associated with Abhinav Health Care Products Pvt Ltd as R & D team.

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Figures

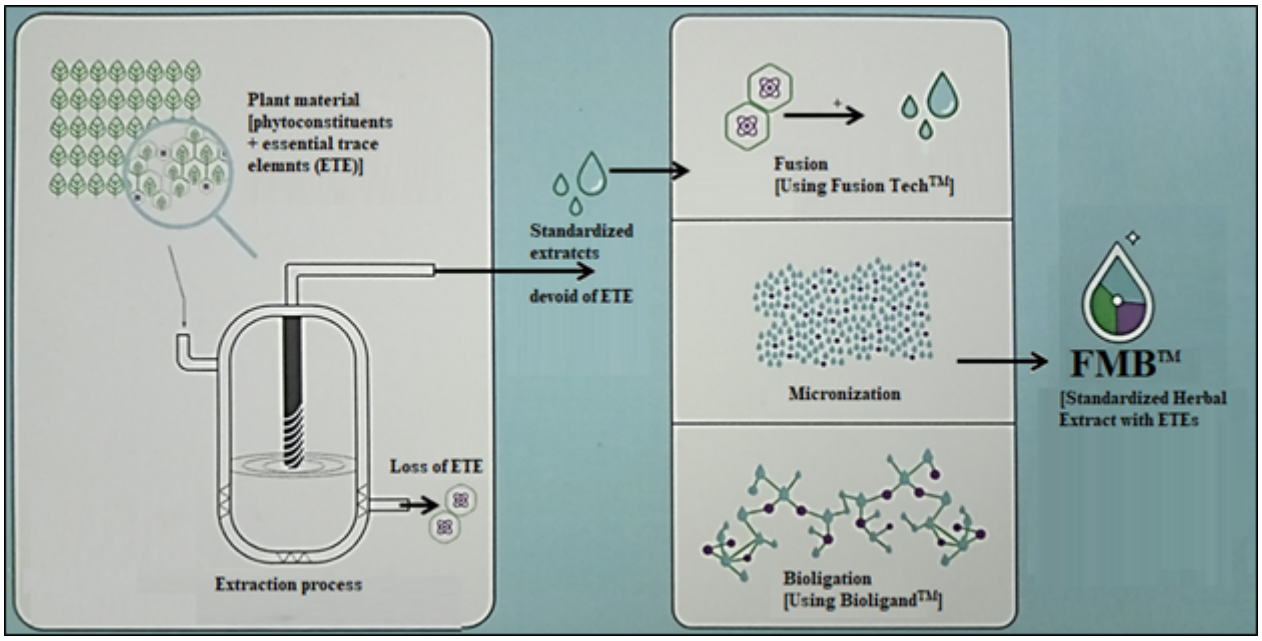


Figure 1

Typical flow process of FMB techniques to develop the novel herbal extract.

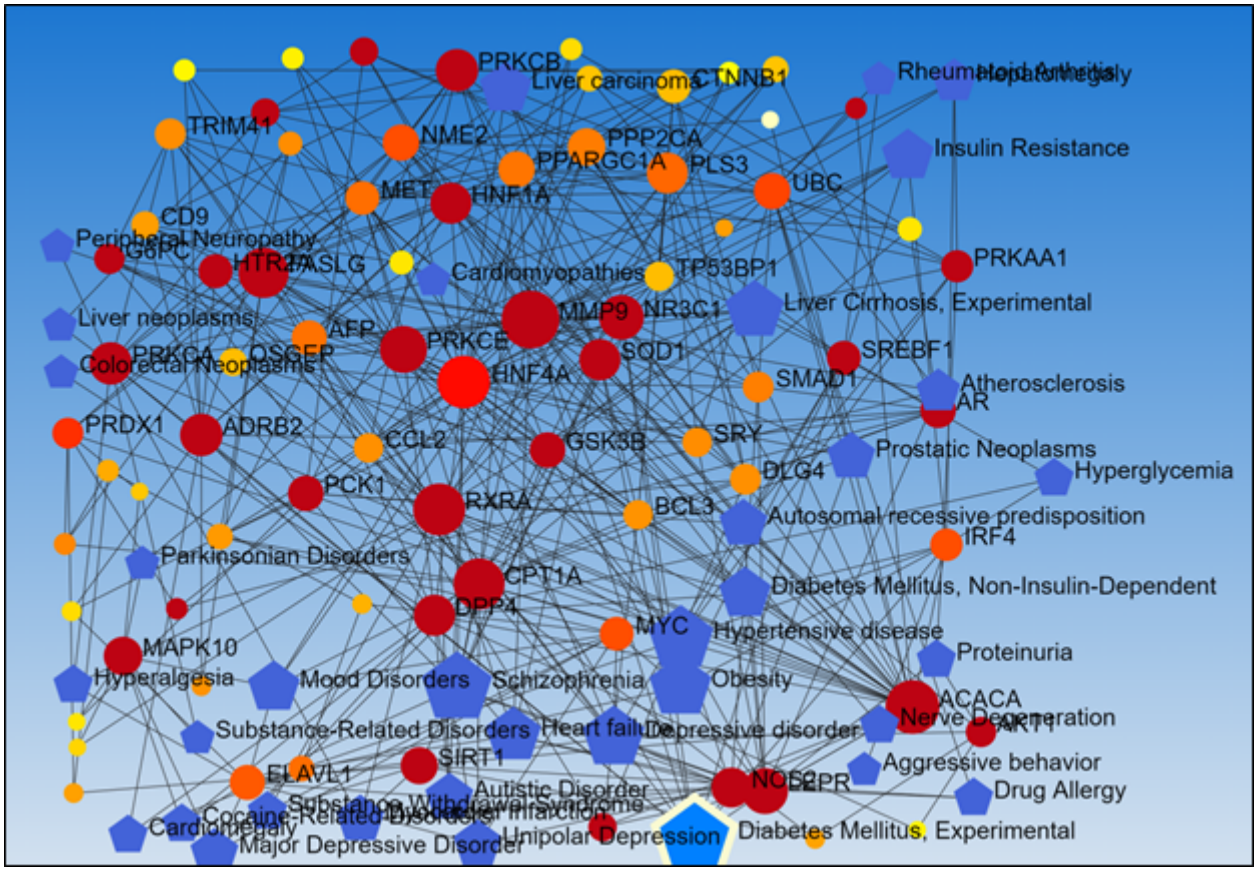


Figure 2

Targets-disease interaction analysis. The blue colour represents the disease and the rest shows the target genes.

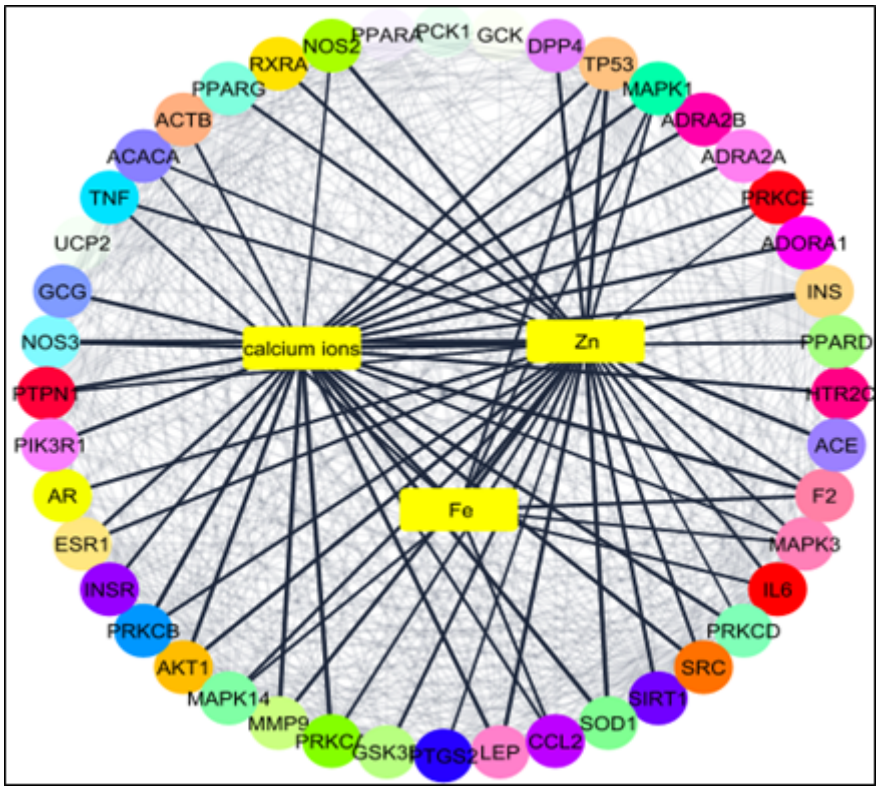


Figure 3

Interactions of diabetes-related proteins and essential trace elements.

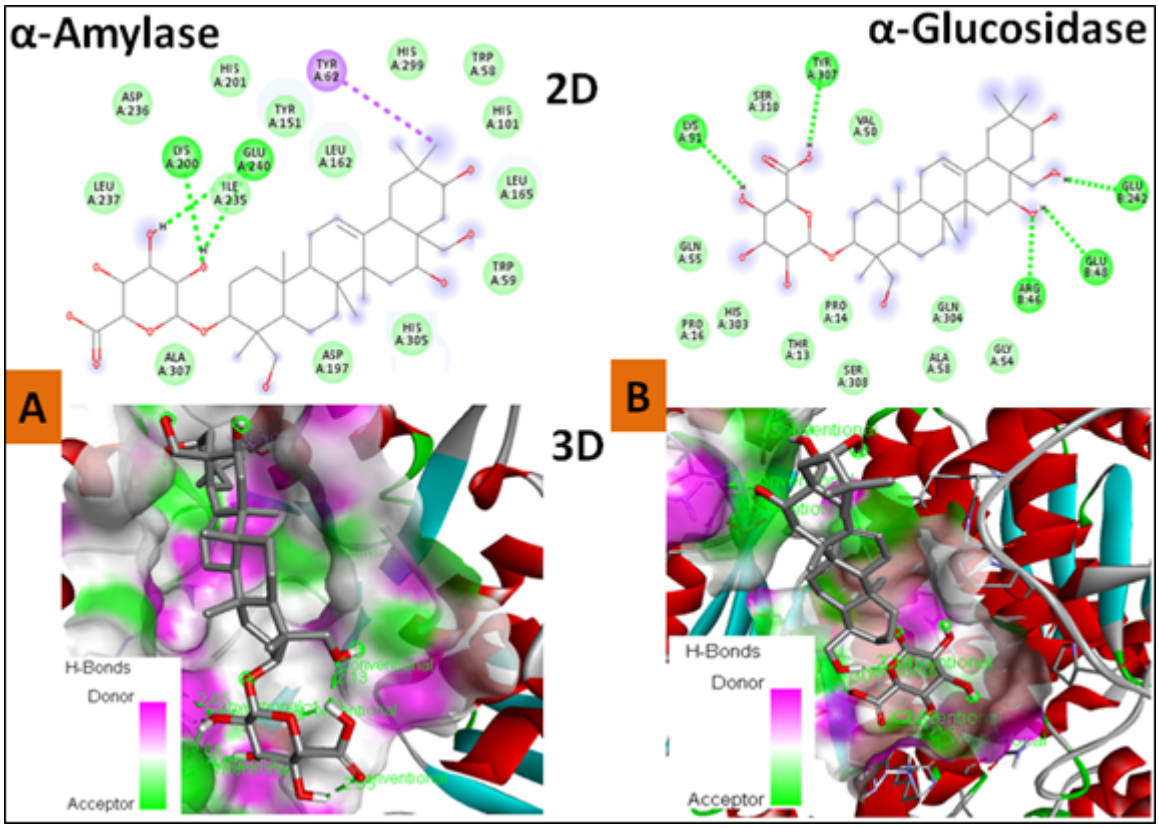


Figure 4

2D and 3D view of the binding interaction of gymnemic acid with α-amylase (A) and α-glucosidase (B), respectively.