TITLE: *In-Vitro* and *In-Sillico* antidiabetic propensities on evaluating the mode of action and health risk assessment of commercial antidiabetic polyherbal formulations

INTRODUCTION

Diabetes mellitus (DM) is a disorder of multiple aetiologies characterized by high blood glucose with abnormal carbohydrate, protein, and lipid metabolism(Srivastava et al., 2017). Absence or/and insensitivity of insulin leads to accumulation of glucose in the blood, causing various secondary macro-vascular and micro-vascular complications (Lankatillake et al., 2019). The prevalence of DM is predominantly increasing due to sedentary lifestyles and the consequential upsurge in obesity. It has been estimated that about 171 million people worldwide suffer from DM. Accounted for 5 million deaths worldwide in 2017 which stances a huge challenge in developing countries because of its inadequate resources(Cho et al., 2018).

Oral hypoglycaemic agents, insulin, and combinatorial approach are presently available pharmacotherapies for management of DM. Drawbacks of present therapies include toxic side effects and prolonged use leading to diminution (Choudhury et al., 2018). With the associated side effects and limitations of present therapies, continuous research on natural sources is being carried out to develop new formulations for effective management of diabetes and its related complications (Gupta et al., 2017).

Ayurveda originated in India 2000 years ago, has a wide range of use in human healthcare. World health organization (WHO) recommends the use of polyherbal formulation (PHF) in the national health care system as an emerging therapy for various diseases (WHO 2010). India is considered as the diabetic capital of the world and therefore to overcome the challenge, antidiabetic polyherbal formulation (APH) can be considered as a contender to resolve the present scenario as the synergistic effect of the herbs present in APH achieves efficiency in multidirectional approach. Literature repots plant bioactive compounds exhibiting digestive enzyme inhibition abilities with their capability to bind to enzyme protein (Ojo et al., 2019; Thengyai et al., 2019). Additionally, dietary fibers and their gelatinous polysaccharides play a major role in the reduction of postprandial plasma glucose levels in diabetes mellitus. Various previous reports have shown that appropriate glycemic control reduced the prevalence of retinopathy, nephropathy, and neuropathy, etc(M. A. Bhutkar et al., 2017; López et al., 1996; Sairam & Urooj, 2012).Literature have revealed the potential APH in reducing blood glucose levels on streptozotocin induced diabetic rats, whereas lacks exhaustive molecular mechanism of action for the observed activity(Mandlik et al., 2008). Systemic scientific evaluation of

plants and formulations derived from them have proposed herbal formulation as a promising avenue against synthetic formulation(Lankatillake et al., 2019) .

Available synthetic remedies include insulin analogues, sulphonylureas, biguanides, dipeptidyl peptidase-4 inhibitors, thiazolidiones, α-glucosidase inhibitors, etc, which is widely studied for their mode of action in management of DM(Choudhury et al., 2018).On the other hand assessing the mechanism of action of APH is a cumbersome process due to the large number of bioactive compounds present (Pereira et al., 2019). Therefore, use of computational biology may resolve the process, further making it cost effective(Shreenithi et al., 2019). The lock and key mechanism of protein and ligand can be observed by *in-silico* approaches, providing the details of amino-acids and binding forces(Bharathi et al., 2014). Previous reports on various polyherbal formulation have revealed the compounds responsible for synergistic mechanism of action using *in-silico* approaches(Liu et al., 2017). Further various drug have also been developed and approved by FDA which was prepared using the advanced computer-aided drug design methods, few examples are Dozamide (Approved by FDA in 1995), Imatinib (Approved by FDA in 2001), Dasatinib (Approved by FDA in 2006), and Ponatinib (Approved by FDA in 2012) etc (Yi et al., 2018).

Additionally with the expanding demand, there is raising concern with the safety and efficacy of APH (Choudhury et al., 2018). Herbal formulations have shown lack of safety assessment in previously reported studies (Archibong et al., 2017; Choudhury et al., 2018). A study conducted on APH sold in the market of Ghana was found to be surpassing the safety limits (Mensah et al., 2019).

Systemic scientific evaluation of plants and formulations derived from them have proposed herbal formulation as a promising avenue against synthetic formulation(Lankatillake et al., 2019) .Our study intends to prove the mechanism of action exerted by the APH in management of DM by using various in-vitro and in-sillico approaches. In-vitro studies includes glucose adsorption, diffusion, amylolysis kinetics and transport across the membrane of yeast cells. Followed by in-sillico analysis on DPPiv and ppr-gamma inhibition by the APH by molecular docking and molecular dynamic simulation. Further evaluated the concentrations of 35 essential elements, trace elements, and heavy metals present in selected six commercial APH and also to appraise the associated health risks which would assure the pre-market and post-market quality of APH available in the Indian market.

Identifying the safety levels and the mode of action of the polyherbal formulation can reform the crude formulation and also make it globally acceptable(Butala et al., 2017)

Objectives –

- To asses the APH for the concentration of Macro, Trace-elements and heavy metal present followed by its health risk assessment.
- To investigate *in vitro* mechanism of anti-diabetic activity by inhibition of glucose diffusion, adsorption, amylolysis kinetics and uptake by yeast cell
- To identify the bioactive compounds by LC-MS/MS
- To assess the *in-silico* mechanism -based studies of the formulations which exhibited better activity

Material and Methods –

Chemicals and reagents

Antidiabetic polyherbal preparation were purchased from local Indian market. Glucose oxidase peroxidase reagent was procured from Agappe Diagnostic Ltd, India. Dialysis bags (12,000 MW cut-off) were used from Himedia laboratories, India. All other solvents and chemicals used were of analytical grade and were obtained from commercial sources.

Sample Collection

Six widely used APH were purchased from the Indian market and they were randomly coded: BG, DB, DT, MA, MH, and SN (Table 1: Composition).

Preparation of extract

Selected antidiabetic polyherbal preparation (5g) was extracted with 150ml of solvent for 24 h by cold maceration technique. The 70% hydro-alcoholic extract (HAE) and Aqueous extract (AQE) were filtered with Whatman filter paper #1. Filtrate was concentrated, stored in sterile vials at 4°C for further analysis (Telapolu et al., 2018).

Metal analysis by Handheld X-ray spectrophotometer (HH-XRF):

Hand held XRF (HHXRF) is the need of the hour to analyze metals, powders and alloys. HH-XRF was used as other XFR technique was cumbersome and difficult to handle. To test the efficiency of the HHXRF 6 powders of antidiabetic polyherbal formulation was analyzed to detect elements. The sample was powdered finely and was placed in a cubical box for irradiation by X-ray tube (Rhodium tube was used for irradiation). The spectrum was obtained

in 20 seconds. The beam lines were obtained of (Beam 1 from 12 to 36 keV) and Beam 2 from 0-12 KeV) (Denni et al., 2019).

Health risk assessments

Health risks assessment of elements in APH can be projected by the methods which are used for assessing carcinogenic or noncarcinogenic risks. Estimated daily intake (EDI) and target hazard quotient (THQ which is introduced by the US Environmental Protection Agency for noncancer risk assessment (USEPA 1989, 1992, 1993; Zhu et al., 2013).

Estimated daily intake of the heavy metals

The estimated daily intake (EDI) of each element present in the APH was determined by the following equation which is recommended by US-EPA:

$$\mathrm{EDI} = \mathrm{ED} \times \mathrm{C} \div \mathrm{W_{AB}}$$

Where; EDI is the estimated daily intake of the elements, C is the determined macro-elements, trace-elements and heavy metals content in the APH, ED is the daily dosage of the APH (Table 1) and W_{AB} is the Indian average body weight; (70 kg adults) (Mensah et al., 2019).

Target hazard quotient (THQ)

The human health hazard posed by element exposure is assessed by target hazard quotient (THQ), which is the ratio of the average EDI to the reference dose (RfD) for an individual pathway and chemical.

$$\mathrm{THQ} = \mathrm{C} \times \mathrm{IR} \times \mathrm{EF} \times \mathrm{ED} \div \mathrm{BW} \times \mathrm{AT} \times \mathrm{RfD}$$

Where, IR: ingestion rate (dosage of the formulation/day); EF: exposure frequency (365days/year); ED: exposure duration (35 years) equivalent to vulnerable age of diabetic population subtracted from average lifespan which is considered as 70 years; BW: body weight in kilogram; and AT is the average time for non-carcinogens (365days/year ×number of exposure years, assuming 70 years). International oral reference dose values for the elements RfDo (mg kg−1 day−1). The reference values as stated by FAO/WHO or previously published literature material (Institute of medicine, food and nutrition board 1997, 2010; Mensah et al., 2019).

Glucose adsorption capacity

Antidiabetic polyherbal preparation extracts (250 mg) were added to glucose solution (25 mL) in logarithmic concentrations (5, 10, 20, 50 and 100 mM), incubated at 37°C for 6 h. The reaction mixture was centrifuged at 4,000 x g for 20 min followed by estimation of glucose in the supernatant by using glucose oxidase- peroxidase method, Absorbance was read at 520nm, Acarbose was taken as positive control (Paul & Majumdar, 2020; Sayyed & Wadkar, 2018). Bound glucose was calculated with the following equation:

$$\text{Bound Glucose} = \frac{(G\,1 - G6) \times \text{Volume of solution}}{\text{Weight of the extracts}}$$

G1: Glucose content initially

G6: Glucose content after 6 hrs incubation.

Glucose diffusion

Glucose solution 20mM (25ml) was added to antidiabetic polyherbal preparation extracts (0.25g), the mixture was dialyzed against 200ml of distilled water at 37°C. Followed by estimation of glucose in the dialysate at 30, 60, 120 and 180 min interval using glucose oxidase peroxidase kit (M. Bhutkar & Bhise, 2013a; Paul & Majumdar, 2020). Control test was performed without extract, Acarbose was taken as standard. Glucose dialysis retardation index (GDRI) was calculated with the following equation.

$$\text{GDRI (\%)} = \frac{(100 - \text{Glucose content with the addition of extract})}{\text{Glucose content of the control}} \times 100$$

Amylolysis kinetics

To 1% antidiabetic polyherbal preparation extract 0.4% of α-amylase and 4% starch solution 25ml was added and was dialysed against distilled water (200ml) at 37°C (pH-7). The glucose concentration was estimated in the dialysate at various time intervals 30, 60, 120 and 180 min and a control test without addition of the extract was also performed (Ou et al., 2001; Paul & Majumdar, 2020)

Glucose uptake by yeast cells

Commercial baker's yeast (EasyGrow Baker's) was washed in distilled water with repeated centrifugation (3,000 x g; 5 min) till a clear supernatant was obtained; further 10% (v/v) suspension was prepared with the same. Different concentrations of both the extracts (1-5 mg) were added to 1 mL of glucose solution (5-25 mM), mixture was incubated for 10 min at 37 ◦C. Reaction was initiated by adding 100µL of yeast suspension, vortexed and incubated at 37 °C. After 60 min, tubes were centrifuged (2500 x g, 5 min) and glucose was estimated in the supernatant. Precent increase in glucose uptake by yeast cells was calculated using the following formula (Cirillo, 1962; Sayyed & Wadkar, 2018).

$$\text{Percent Increase glucose uptake} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

Bioactive compounds identification MA

Bioactive compounds that consisting the formulations were identified by LC-MS/MS analysis. The compounds were sequestered with existing reports on DM, further selected based on less/no reports on targeted proteins i.e., Dipeptidyl peptidase-IV (DPPiv; PDB-ID 1J2E), and peroxisome proliferators-activated receptor gamma (ppr-gamma; PDB-ID 2ZK0)(Banerjee et al., 2018; Oso et al., 2020).

Physicochemical analysis, Lipinski rule and ADMET properties

Selected compounds were screened if they abide by the Lipinski rule from PubChem database (www.pubchem.ncbi.nlm.nih.gov). Followed by Physicochemical analysis, and ADMET properties of the selected compounds using SWISS ADME server (http:// www.swissadme.ch/index.php) by means of associated SMILES (Daina et al., 2017).

ProTox analysis

Toxicity profile grounded on 33 models' identification of several toxicity endpoints i.e., acute toxicity, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity, adverse outcomes (Tox21) pathways and toxicity targets of the selected compounds were computed using ProTox-II (tox.charite.de)(Banerjee et al., 2018).

Protein preparation

3D structure of the proteins such as Dipeptidyl peptidase-IV (DPPiv; PDB-ID 1J2E) and peroxisome proliferators-activated receptor gamma (ppr-gamma; PDB-ID 2ZK0) were derived

from Protein data bank. The protein was complexed with, peptide inhibitor, water molecules, inhibitor, and other heteroatoms, hence modified protein molecule i.e., removing the above stated molecules was used for docking(Kiran et al., 2020).

Molecular docking

Molecular docking of the selected ligands with targeted proteins for DM and associated complication was assessed. PyRx software was used by AutoDock Vina for molecular docking (e Scripps Research Institute, La Jolla, CA, USA). (e docking Graphical User Interface (GUI) frontend PyRx version 0.8 (https://pyrx.sourceforge.io/) (e Scripps Research Institute, La Jolla, CA, USA) was incorporated to prepare all protein and ligand files for docking and for the generation of docking parameter input files.

PyRx was employed to convert all protein and ligand PDB files into PDBQT format. Protonation states for titratable sidechains of the protein were based on those assigned using OpenBabel (OpenEye Scientific Software, Santa Fe, NM, USA) at pH 7. OpenBabel was used to run energy minimization for selected or all molecules. Docking boxes were set using the "maximise" option in PyRx around the protein receptor in order to enable "blind" docking, in which the entire protein surface and inner compartments were made accessible for potential binding of ligands.

Additionally, Bio-Hpc webserver blind docking was incorporated(https://bio-hpc.ucam.edu/achilles/dashboard) . Bio-Hpc validation of molecular docking poses and support our Pyrex docking (Oso et al., 2020)

Molecular Dynamic Simulation

Conformational stability and interaction of the two better binding affinity of the Docked complexes were evaluated incorporating MDS using iMODS server (http://imods.chaconlab.org) by normal mode analysis (NMA) predicting properties such as deformability, mobility profiles, eigenvalues, variance, co-variance map and elastic network of the protein-ligand interactions. The steps in iMOD server includes, Click on "Basic" from the navigator of the web server. Followed by upload the docked and submit the docked complex for simulations(Oso et al., 2020).

Statistical Analysis

Values are represented as mean±SE (n=3). Significant differences were analyzed using Two-way analysis of variance (ANOVA), with Tukey's multiple comparisons test at $P<0.05$. Analysis and Figure preparations were carried-out in GraphPad prism 6.

Result and Discussion –

TABLE 1: Composition of different antidiabetic herbal polyherbal formulations

Sample	Composition
BG	*Berberis aristata* DC, *Pterocarpus marsupium* Roxb, *Gymnema sylvestre* (Retz.) R.Br. ex Sm, *Rubia cordifolia* L, *Trigonella foenum-graecum* L, *Tinospora cardifolia Miers*, *Berberis Aristata* and *Tinospora cordifolia*
DB	*Gymnema sylvestre*, *Pterocarpus marsupium*, Yashtimadhu, *Glycyrrhiza glabra*, *Casearia esculenta*, *Syzygium cumini*, *Asparagus racemosus*, *Boerhavia diffusa*, *Sphaeranthus indicus*, *Tinospora cordifolia*, *Swertia chirata*, *Tribulus terrestris*, *Phyllanthus amarus*, *Gmelina arborea*, *Gossypium herbaceum*, *Berberis aristata*, *Vidangadi lauham*, *Momordica charantia*, *Piper nigrum*, *Ocimum sanctum*, *Abutilon indicum*, *Rumex maritimus*, *Curcuma longa*
DT	*Syzigium cumin*, *Plectranthus Amboinicus*, *Andropogan Muricatus*, *Cinanamum zeylanicum*, *Anacyclus Pyrethrum*,*Cassia fistula*, *Strychnos potatorum*, *Cocculus cordifolius*, *Gymnema sylvestre*
SN	*Vachellia nilotica*, *Tinospora cordifolia*, *Catharanthus roseus*, *Momordica charantia*, *Andrographis pinculata*, *Gymnema sylvestre*, *Trigonella foenum-graecum*, *Azadirachta indica*, *Ficus racemosa*, *Ocimum tenuiflorum*, *Phyllanthus niruri*, *Cinnamomum verum*, *Curcuma longa*, *Picrorhiza kurrooa*
MA	Momordica charantia , Syzygium cuminii, Mangifera indica, Gymnema sylvestre
MH	Pterocarpus marsupium, Salacia reticulata, Curcuma longa, Emblica officinalis, Momordica charantia, Tinospora cordifolia

Result and Discussion

Heavy metal analysis

Six commercial APH were assessed for thirty-five elements (Mg, Al, Si, P, S, K, Ca, Ti, Mn, Fe, Zn, Rb, Sr, Zr, Nb, Mo, Cd, Th, Cr, Pb, Bi, U, V, Co, Ni, Cu, As, Se, Y, Ag, Sn, Sb, Ba, W, and Hg), representing the data in ppm (Table 2) and Fig 1A-1F represents the X-Ray spectrum of the APH by HH-XRF (Beam 1: 12 to 36KeV and Beam 2: 0-12KeV). Concentrations of metals detected in six tested formulations varied significantly, which can be due to different geochemical characteristics of soil from which the plants are grown and used for preparing the APH. Additionally, types of herbs used and the different parts of plants including the tubers, leaves, seeds etc. (Table 1) might play an important role for the vast range of concentrations of elements (Rao et al., 2011). The average concentration of elements in descending order is represented (Table 3). V, Co, Ni, Cu, As, Se, Y, Ag, Sn, Sb, Ba, W, and Hg were not detected in any tested APH, hence, can be concluded that these formulations are devoid of toxicities arising from the above elements.

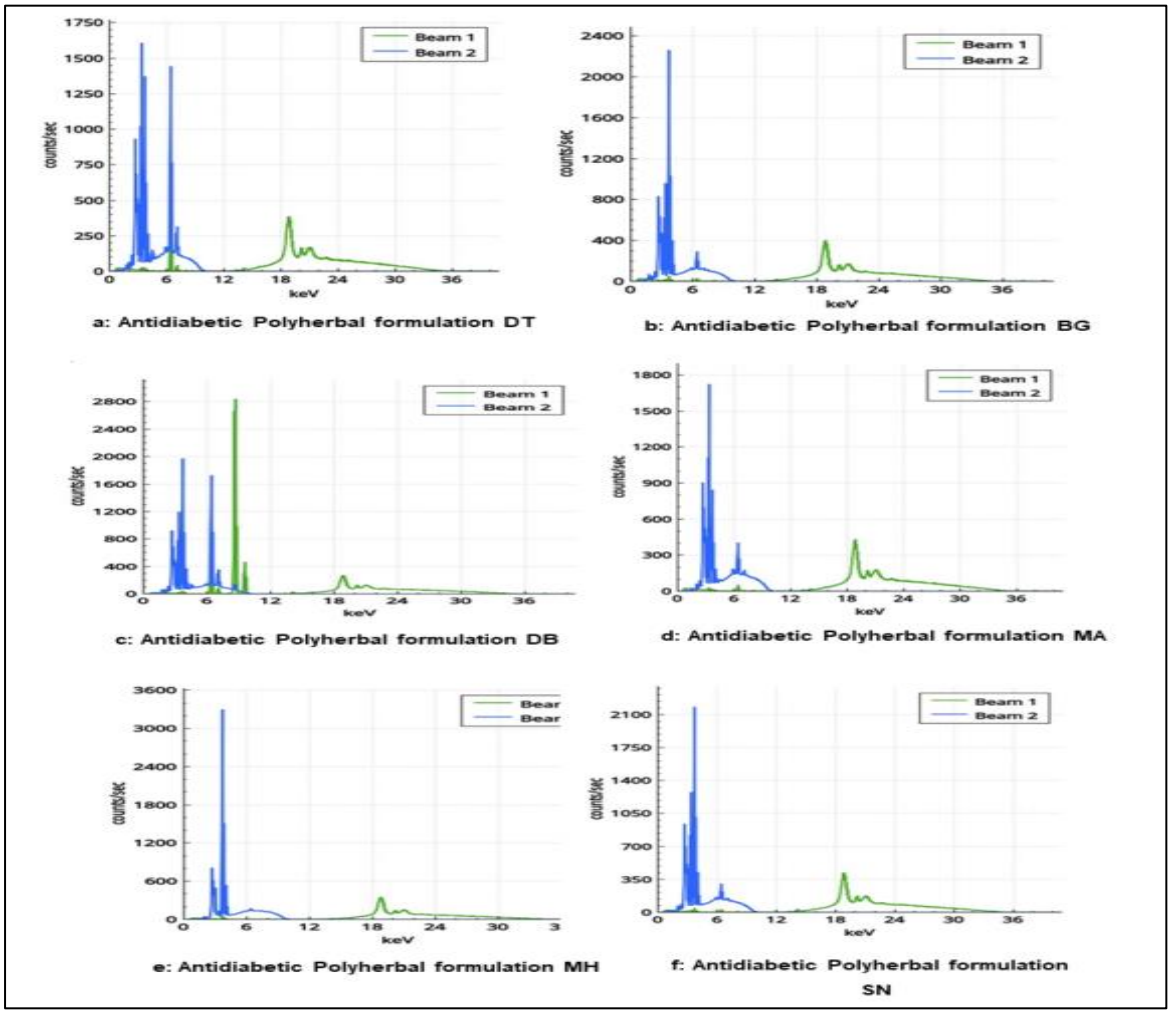

Fig 1 a- f, X-Ray spectrum of Six Antidiabetic Polyherbal formulations by HH-XFR (Beam1: 12 to 36KeV and Beam2: 0-12 KeV)

Table2 Element analysis of Six APH in ppm (Note: Anything beyond 10000 ppm is represented in % and ND represents not detected)

Elements	DT	DB	BG	MA	MH	SN
Mg	1.24%±0.92	ND	ND	ND	ND	ND
Al	2900±1200	1300±1100	ND	1400±1200	ND	1500±1100
Si	5860±370	7889±410	8960±440	1280±250	ND	430±220
P	2150±120	2150±120	1420±110	2080±120	780±120	2330±130
S	1452±91	978±89	1128±85	803±84	ND	507±85
K	1.36%±0.024	9520±170	8360±140	1.48%±0.021	632±62	1.02%±0.016
Ca	7610±140	1.10%	1.39%±0.019	4454±83	2.31%±0.033	1.25%±0.017
Ti	520±250	220±220	240±230	ND	ND	ND
Mn	141±33	114±29	94±30	137±32	28±26	76±28
Fe	5140±140	8740±190	633±46	954±53	100±25	659±46
Zn	23±5	1.56%±0.025	18±5	20±4	21±5	26±5
Rb	8±2	15±2	6±2	10±2	3±2	7±2
Sr	18±2	44±3	13±2	10±2	6±2	25±2
Zr	7±4	15±4	ND	8±3	4±4	5±3
Nb	4±3	ND	5±3	4±3	9±3	3±3
Mo	7±5	ND	6±5	ND	7±5	ND
Cd	30±17	ND	22±17	32±16	ND	31±16
Th	10±9	ND	ND	ND	22±10	ND
Cr	ND	ND	ND	ND	30±18	ND
Pb	ND	7±4	ND	ND	ND	7±4
Bi	ND	48±12	ND	ND	ND	ND
U	ND	ND	ND	ND	4±4	ND

Following trace elements (Al, Mn, Fe, Zn, Mo, Cr, Si, Ti, Rb, Sr, Zr, Nb, Cd, Th, Pb, Bi, and U) were analyzed in the tested formulations. The toxicity levels of the trace elements detected in the formulations were assessed by EDI (Table 3) and THQ (Table 4) to analyze the risk associated with the daily intake of the elements for a prolonged period of time (USEPA, 1989). Though most of the elements detected were observed within the permissible range, it fails to give an overview of any health threat hence, the EDI and THQ were also analyzed. The EDI value gives an account of the elements ingested, according to the body weight and exposure period based on the daily dosage of APH. THQ reports the assessment of health risks associated with consumption of the calculated EDI (USEPA, 1989). In the present study, THQ of all elements were lower than unity except for Rb in MA which is in accordance with the previously reported study (Mensah et al., 2019).

Table 3 Estimated daily intake of the elements detected in the six Antidiabetic Polyherbal formulations

Elements	DT	DB	BG	MA	MH	SN
Mg	292571	ND	ND	ND	ND	ND
Al	82857	37143	ND	300000	ND	128571
Si	167429	225400	279496	274285.7	ND	36857
P	61429	61429	50308.6	445714	11142.8	199714
S	41485.7	32228.6	34649.1	172071	ND	43457
K	296000	272000	296182.9	2245714	9028.6	858857
Ca	217429	288571	368102.8	954428.6	290143	878571
Ti	14857	6857.1	7794.3	ND	ND	ND
Mn	4029	3257	3330.3	29357	400	6514
Fe	146857	249714	22426.3	204429	1429	56486
Zn	657	445714	637.7	4286	300	2229
Rb	229	429	212.6	2143	42.8	600
Sr	514	1257	460.6	2143	85.7	2143
Zr	200	429	ND	1714.3	57	429
Nb	114	ND	177.1	857.1	128.6	257
Mo	200	ND	212.6	ND	100	ND
Cd	857	ND	779.4	6857	ND	2657

Th	286	ND	ND	ND	100	ND
Cr	ND	ND	ND	ND	429	ND
Pb	ND	200	ND	ND	ND	600
Bi	ND	1371.4	ND	ND	ND	ND
U	ND	ND	ND	ND	57	ND

Table 4 Target Hazard quotient (Risk level >1)

Elements	DT	DB	BG	MA	MH	SN
Mg	0.0005	ND	ND	ND	ND	ND
Al	0.0064	0.0289	ND	0.156	ND	0.01
P	6.80E-05	0.0007	5.50E-05	0.0033	2E-04	0.0002
S	7.10E-05	0.0005	5.90E-05	0.002	ND	7.40E-05
K	7.60E-05	7E-04	7.60E-05	0.004	4.60E-05	2E-04
Ca	0.0002	0.002	3E-04	0.0049	0.005	7E-04
Ti	2.30E-05	0.0001	1.20E-05	ND	ND	ND
Mn	0.001	0.011	0.001	0.067	0.003	0.002
Fe	0.014	0.243	0.002	0.133	0.003	0.006
Zn	4.60E-05	0.315	4.50E-05	0.002	4E-04	2E-04
Rb	0.034	0.629	0.031	2.108	0.126	0.088
Sr	7E-04	0.016	6E-04	0.019	0.002	0.003
Nb	1E-04	ND	0.004	0.007	0.003	3E-04
Mo	0.003	ND	0.01	ND	0.035	ND
Cd	0.002	ND	0.002	0.119	ND	0.007
Cr	ND	ND	ND	ND	0.333	ND
Pb	ND	0.389	ND	ND	ND	0.117
U	ND	ND	ND	ND	0.741	ND

The percentage contribution of the elements detected is represented in Fig 2. Among all elements, the maximum percentage of Th, Cr, and Pb were detected in DT, MH, and SN

respectively whereas Rb accounts for the highest percentage in DB, BG, and MA and hence can be concluded as major contaminants in the respective APH.

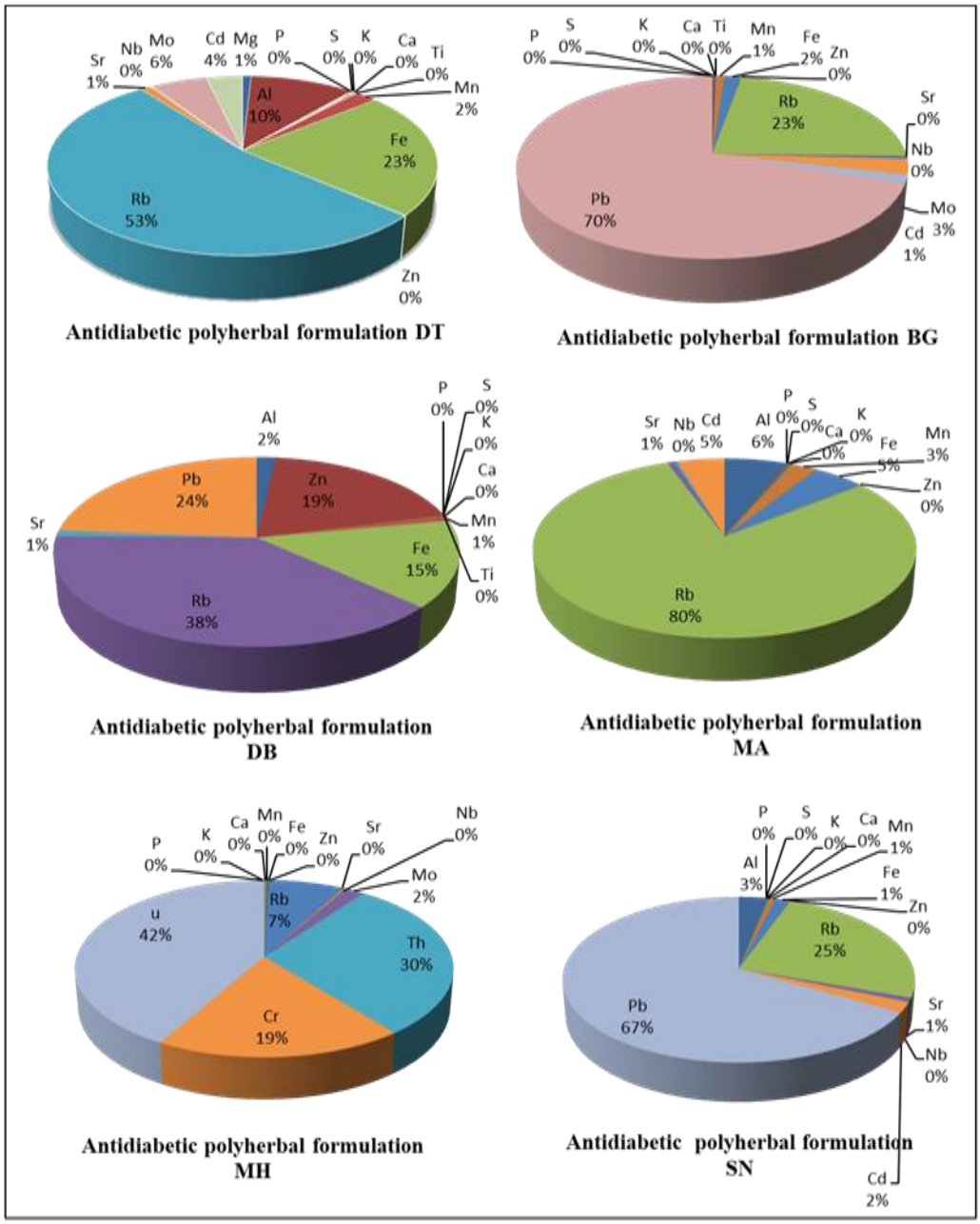

Fig 2 Percentage contribution of each element to the Target Hazard Quotient of six Antidiabetic Polyherbal formulations

Effect of extracts on glucose adsorption capacity

HAE and AQE extracts of 6 selected APH were assessed for their glucose adsorption capacity. It was observed that with an increase in glucose concentration there was an increase in bound glucose (Figure 3). Adsorption capacity of the extracts is directly proportional to the molar

concentrations of glucose. Maximum glucose binding was observed at highest concentration by MA i.e., 84.48±1.02 mM and 71.14±1.09mM respectively at 100mM concentration for HAE and AQE respectively. Adsorption capacity of extracts might be due to presence of soluble fibres, insoluble fibres and bioactive constituents. Similar interpretations were reported for insoluble fiber-rich fractions isolated from Averrhoa carambola(Chau et al., 2004).

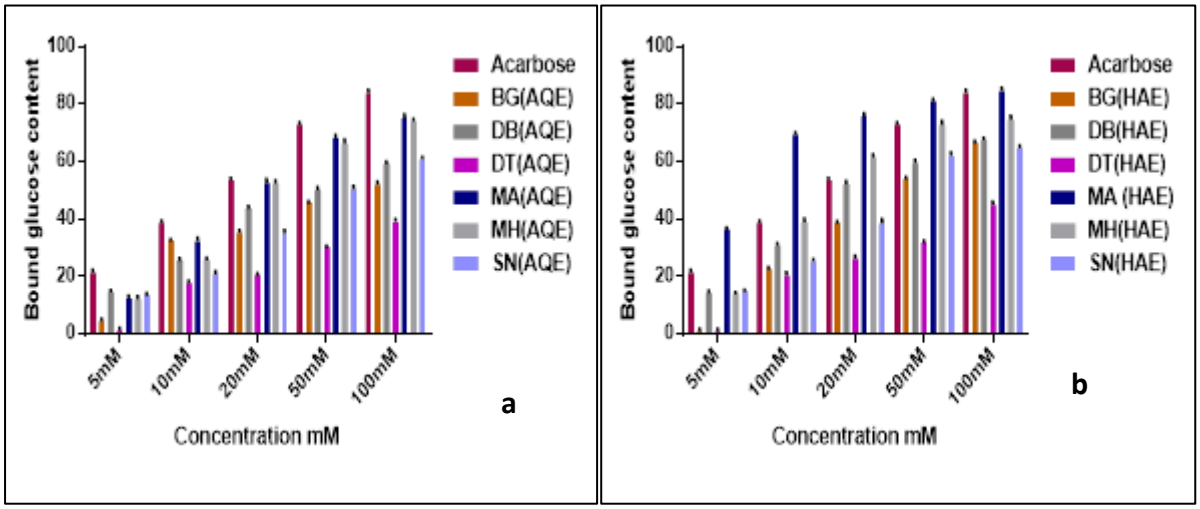

Figure 3: Glucose binding capacity of a: HAE and b: AQE at different concentrations of glucose. Values are mean ± SE of triplicate determinations.

Effect of extracts on in-vitro glucose diffusion

In-vitro glucose dialysis retardation index (GDRI) is a convenient method to analyse the effect of extracts on interruption of glucose adsorption in gastrointestinal tract (Bhutkar et al., 2017; Harish et al., 2014). All the HAE and AQE extracts exhibited significant inhibitory effects on movement of glucose into external solution when compared to control (Table 5). The rate of glucose diffusion across the dialysis membrane was found to be directly proportional with time. The GRDI reduced over time for both the extracts with highest values observed at 180min. HAE exhibited similar GRDI when compared with standard Acarbose. Our results corroborate with the studies on different samples where GRDI reduced over time (Ahmed & Urooj, 2010; Ou et al., 2001). Previous studies has proposed several possible mechanism for lowering of glucose which might be due to adsorption or inclusion of the smaller glucose molecules into the structure of particles present in the extracts. Another possible explanation for glucose diffusion ability might be the capability of polysaccharides forming gelatinous compounds in aqueous solution, which decreases glucose availability in small intestine, resulting in reducing postprandial glucose levels (Ahmed & Urooj, 2015; Gallagher et al., 2003; López et al., 1996).

Table 5 Effect of extracts on glucose diffusion and GDRI.				
	Glucose content in dialysate (mM)			
Time (min)	30	60	120	180
Control	0.929	1.313333	1.503333	1.947
MA(HAE)	0.68±0.071(27.88%)	1.12±0.083 (14.97%)	1.28±0.044 (14.63%)	1.36±0.05(14.39%)
MA(AQE)	0.73±0.05 (21.74%)	1.07±0.07 (18.32%)	1.32±0.02 (11.99%)	1.5±0.01 (13.82%)
MH(HAE)	0.67±0.04 (27.45%)	1.063±0.05 (19.09%)	1.28±0.04 (14.63%)	1.49±0.04(14.68%)
MH(AQE)	0.77±0.06 (17.44%)	1.21±0.05 (7.67%)	1.39±0.07 (7.007%)	1.5±0.01(13.88%)
BG(HAE)	0.708±0.05 (23.79%)	1.09±0.47 (17.38%)	1.24±0.05 (17.25%)	1.524±0.02(13.99%)
BG(AQE)	0.807±0.12 (13.24%)	1.24±0.04 (5.36%)	1.44±0.05 (3.81%)	1.51±0.24 (13.87%)
DB(HAE)	0.76±0.04 (18.29%)	1.14±0.04 (12.97%)	1.27±0.04 (15.45%)	1.56±0.02 (13.98%)
DB(AQE)	0.81±0.02 (12.38%)	1.25±0.02 (4.67%)	1.44±0.03 (3.68%)	1.5±0.004 (13.99%)
DT(HAE)	0.82±0.04 (11.41%)	1.19±0.02 (8.86%)	1.44±0.04 (4.54%)	1.53±0.03 (11.84%)
DT(AQE)	0.9±0.03 (2.9%)	1.3±0.04 (0.71%)	1.49±0.03 (0.69%)	1.53±0.02 (8.63%)
SN(HAE)	0.77±0.02 (17.00%)	1.19±0.04 (9.47%)	1.36±0.06 (7.87%)	1.53±0.03 (11.84%)
SN(AQE)	0.84±0.04 (9.69%)	1.27±0.03 (3.68%)	1.45±0 (3.55%)	1.54±0.009(11.75%)

Effect of extracts on in-vitro amylolysis kinetics

The rate of glucose diffusion were analysed at every 30 min interval, diffusion rate was nil at 30min i.e., 100%. Further from 60 min the rate of glucose diffusion reduced significantly ($p \leq 0.05$), highest GDRI was observed at 180min. Present analysis revealed that GRDI value reduced gradually as time increases which is in comparable with previous reports (Bhutkar et al., 2017; Harish et al., 2014). MA and MH (HAE) reduced the total glucose movement from 27.88% and 27.45% to 14.39% and 14.68% at 180 min, which displayed a gradual decrease. Thus, might be considered as a promising inhibitor which inhibits the diffusion of glucose across the plasma membrane into the blood vessel (Konappa et al., 2020). Effect of extracts on in-vitro amylolysis kinetics values obtained are represented in Table 6. Inhibition of α-amylase can be considered as a limiting step for the release of glucose from starch thus retarding glucose diffusion. α-amylase inhibition might be due to various factors such as fibre content, inhibitors on fibres, encapsulation of starch, thereby decreasing availability of starch to the enzyme (Ahmed et al., 2011; Cirillo, 1962; Ou et al., 2001).

Table 7 Effect of extracts on starch digestibility and GDRI				
	Glucose content in dialysate (mM)			
	30min	60min	120min	180min
Control	0	0.28±0.005	0.354±0.003	0.446±0.009
MA(AQE)	0(100)	0.148±0.005(47.14%)	0.238±0.038(32.8%)	0.37±0.003 (17.01%)
MA(HAE)	0 (100)	0.119±0.003 (57.5%)	0.223±0.008(11.99%)	0.356±0.007 (20.18%)
MH(AQE)	0.0 (100)	0.134±0.03 (52.5%)	0.228±0.027(34.19%)	0.38±0.07 (14.25%)
MH(HAE)	0.0 (100)	0.152±0.06 (45.7%)	0.24±0.047 (30.86%)	0.364±0.049(18.59%)
BG(AQE)	0.0 (100)	0.145±0.03(48.21%)	0.214±0.02(38.4%)	0.374±0.05(16.26%)
BG(HAE)	0.0 (100)	0.127±0.07(54.64%)	0.206±0.07(40.38%)	0.377±0.05(15.59%)
DB(AQE)	0.0 (100)	0.17±0.07(38.57%)	0.254±0.08(27.1%)	0.375±0.041(16.04%)
DB(HAE)	0.0 (100)	0.145±0.03(48.2%)	0.246±0.07(29.36%)	0.393±0.066 (12%)
DT(AQE)	0.0 (100)	0.927±0.05(29.42%)	0.27±0.05(22.74%)	0.413±0.04(6.18%)
DT(HAE)	0.0 (100)	0.809±0.09(38.4%)	0.236±0.03(32.19%)	0.4±0.03(10.44%)
SN(AQE)	0.0 (100)	0.141±0.03 (49.64%)	0.273±0.05 (22.74%)	0.37±0.03 (17.01%)
SN(HAE)	0.0 (100)	0.137±0.04 (51.07%)	0.195±0.03 (43.76%)	0.317±0.048 (28.89%)

Effect of extracts on glucose uptake by yeast cells

Rate of glucose uptake across the yeast cell can be measured by the remaining glucose content in the medium after defined time interval. A linear uptake of glucose was observed for both the extracts, percent increase in glucose uptake by the yeast cells was found to be inversely proportional to glucose concentration. Glucose uptake decreased with higher molar concentration of the glucose solution. Similar studies were reported with various traditional hyoglycemic plants on glucose uptake by yeast cells(Khan et al., 2017). Previous studies on movement of various sugars and glycosides have suggested its transport across the cell membrane which might be due to presence of stereospecific membrane carriers or mediators further enhancing facilitated diffusion process(M. Bhutkar & Bhise, 2013b; Illiano & Cuatrecasas, 1971). Our results are in accordance with previous reports on medicinal plants(Konappa et al., 2020; Sayyed & Wadkar, 2018).

With the better obtained data for MA it was further considered for LC-MS/MS analysis followed by molecular mechanism studies. Previous studies on MA are reported with

antihyperglycemic activity, which exhibited blood glucose lowering effect in *in-vivo* model, whereas lacked data on its mode of action(Mandlik et al., 2008). Therefore, this is the first in-silico molecular mechanism report on selected compounds from MA against diabetes targets DPP-IV and PPARγ.

The LC-MS/MS analysis of the hydroalcoholic extract of MA reveled presence of various bioactive compounds. These were further screened with available literature for their studies on diabetes managements. Compounds with limited studies on DM management and no reports on DPP-IV and PPARγ were chosen. The compounds were filtered with Physico-chemical, pharmacokinetics properties and Lipinski analysis. To further ensure the safety of these compounds SWISS-ADME and PorTox server was used. The result showed drug likeness of the compounds, it is important to assess the drug like compounds state in human body to analyse its toxicity levels, thus ADMET and oral toxicity analysis is an important step(Banerjee et al., 2018; Daina et al., 2017; Kiran et al., 2020). Therefore, the above properties illustrate the likeliness of the compounds to be orally active.

Molecular docking is an important structure dependent computational technique for predicting binding mode of various protein-ligand theoretically. This mainly depends on the various crucial characteristics of the receptor/protein of interest also its interaction with ligands/ bioactive compounds(Bharathi et al., 2014; Oso et al., 2020).

The observed anti-diabetic activity might be due to the various bioactive molecules regulating the activity of multi-target involve in DM management(Pereira et al., 2019). The present study was carried out to identify the molecular interactions of phytochemicals from APH MA against two proteins of interest i.e., DPP-IV and PPARγ.

Incretin therapy is considered as one of the most advance method in DM management which includes DPP-IV inhibitors(Kumar et al., 2021). Glucose-induced insulin secretion is dependent on GLP-1 which has a short half-life and is rapidly degraded by proteolytic enzyme DPP-IV(Srivastava et al., 2017). Thus, this protein might be considered as one of the crucial targets to manage DM. Results of docking study exhibited effective binding of the selected bioactive compounds to DPP-IV protein, the binding affinity values were obtained by Pyrx. Our data showed Glycyrrhetaldehyde with the best binding affinity, whereas Dantrolene and 6-Hydroxyluteolin also exhibited better values when compared to standard drug Vildagliptin (Table 7) This might be due to a greater number of non-covalent interaction providing less binding affinity. Our results have showed better binding affinity when compared to previous

report on various metabolite from blue corn and black bean in DPP-IV inhibition capacity(Damián-Medina et al., 2020).

Table 7: Biding affinity

Blind Docking Binding affinity		
	DPPiv	Ppr-gamma
	PyRx	PyRx
N-D-Glucosylarylamine	-7.0	-7.4
Dantrolene	-8.4	-8.4
Grandinol	-6.7	-7.6
3-O-Methylgallate	-6.6	-6.2
6-Hydroxyluteolin	-8.9	-8.9
Glycyrrhetaldehyde	-9.7	-8.5
Rosiglitazone		-7.6
Vildagliptin	-7.8	

PPARγ plays a crucial role in maintaining homeostasis for lipid and glucose levels. As high level of lipids is associated with phosphorylation of PPARγ by protein kinase Cdk5 leading to insulin resistance(Guasch et al., 2013; Tyagi et al., 2011). Therefore, making it an important target for DM and obesity associated with DM. Inhibiting the phosphorylation process might be able to manage DM, hence our work also intends to study the effect of selected compounds as PPARγ antagonist(Guasch et al., 2013).

To elucidate the mechanism in terms of affinity of selected compounds with PPARγ molecular docking was performed. Our results revealed 6-Hydroxyluteolin with lowest binding affinity, therefore exhibiting better activity. The data also showed Glycyrrhetaldehyde and Dantrolene with better activity when compared with standard drug Rosiglitazone (Table 7).

Molecular dynamic simulation (MDS) is a computer oriented biological simulation process related to size and time scale, for assessing the actions of atoms and molecules physically. It is

important to understanding proper functioning of the macromolecules under study which depends on its mobility(Lopéz-Blanco et al., 2011; López-Blanco et al., 2014). Further enhancing and validating important details for the protein-ligand association at atomic level. Therefore, in the present study MDS was carried out for the compounds i.e., 6-Hydroxyluteolin and Glycyrrhetaldehyde, which displayed significant binding affinity for both the target proteins.

iMOD server a fast, comprehensible and effective tool was incorporated in the current study for determining structural flexibility and motion using Normal mode analysis (NMA). Previous reports have showed significant correlation of iMOD with classical MDS and experimental data. Output from iMOD server provides details on structural deformity, B-factor, eigenvalues, variance, Co-variance and elastic network(Mahmoodpoor et al., 2010; Shrivastava et al., 2020). Hence concluding that the structure considered for molecular dynamic simulation showed better stability (Figure 4).

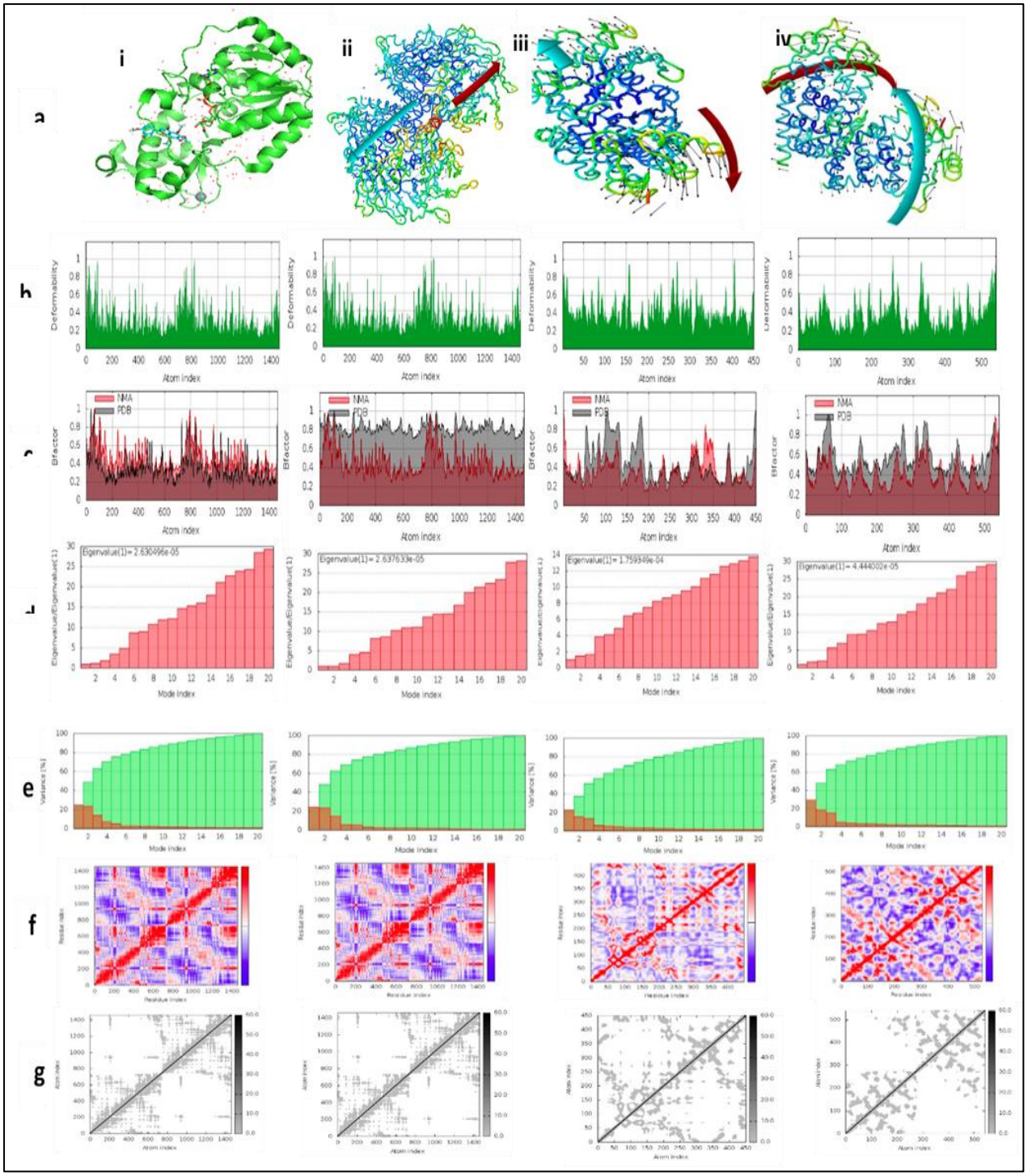

Figure 3 a-f (i-iv): Molecular dynamics simulation of antidiabetic targets (i) DPPiv-6-Hydroxyluteolin (ii) DPPiv- Glycerraldehyde and (iii) ppr-gamma-6-Hydroxyluteolin (iv) ppr-gamma-6-Hydroxyluteolin using iMOD. Protein structure stability was estimated via a) NMA domain mobility b) Deformities of atoms in torsional spaces c) Eigen value d) Variation of atoms due to temperature (B factor) e) Variance f) Covariance map g) Elasticity network.

Impact of the research in the advancement of knowledge or benefit to mankind –

India is one of the epicentres of the global DM pandemic. The number of people with DM in India increased from 26·0 million in 1990 which is expected to increase up to 109.0 million by 2035. Prevalence of diabetes increased in both rural and urban India from 2.4% and 3.3% in 1972 to 15.0% and 19.0% respectively in year 2015-2019, hence presently producing a substantial global economic burden on society. DM and its associated microvascular and macrovascular complications account for most of the morbidity and mortality associated with the disease. DM extends far beyond the classic acute metabolic and chronic vascular complications to increased risk of an ever-increasing array of conditions including Alzheimer disease, cancer, liver failure, bone fractures, depression and hearing loss etc. At present there are different families of oral and injectable drugs available for the treatment of DM which includes sulfonylureas, meglitinides, insulin, TZD and alpha-glucosidase inhibitors, GLP1 receptor agonists, DPP4 and SGLT2 inhibitors etc. Prolonged use of which is associated with various side effects, therefore various established polyherbal formulations are considered as an alternative option. Though these available commercial polyherbal formulations are assessed by *in-vivo* studies they lack systematic mechanistic studies which might increase their acceptability.

Our present work is to elucidate the mechanism of action of various commercial polyherbal formulations by *in-vitro* and *in-silico* approaches. These include hypoglycaemic assessment of the extracts by glucose adsorption, decreasing glucose diffusion rate and at the cellular level by promoting glucose transport across yeast cells. Followed by elemental analysis and health risk assessment of all the selected formulations. The formulation with best activity was further evaluated for their phytochemical identification by LC-MS/MS, followed by *in-silico* molecular docking and dynamics studies on important diabetes targets. This might provide an insight of these selected formulations working in synergistic manner in maintaining blood glucose levels and its associated complications. All the synthetic formulations are studied for their mode of action, hence are accepted. Therefore, our work may validate the claims of these formulations scientifically, thereby additionally increasing their global acceptance and make the formulations a competent for synthetic drugs.

Therefore, with aforementioned study is an important aspect on diabetes which has crippled our country as India which is presently known as “Diabetic capital”.

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