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Research Article

Gas Chromatography-mass Spectrometry Profiling, *In vitro* Antidiabetic, Antioxidant and Antimicrobial Activities of a Novel Polyherbal Formulation

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ABSTRACT

In the present study the methanol extract of a novel polyherbal formulation (PHF) was studied for alpha (α)-amylase and alpha (α)-glucosidase inhibition using an in vitro antidiabetic model. The polyherbal extract was also examined for its antioxidant activity by using free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging method. The study revealed that the polyherbal formulation exhibits potent radical scavenging activity using DPPH as substrate. The methanol extract exhibited significant α-amylase and α-glucosidase inhibitory activities with an IC_{50} value of 31.52 ± 0.74 µg/mL and 53.13 ± 0.97 µg/mL respectively and well compared with standard acarbose drug. Further, the antibacterial activity of methanol extract of PHF evaluated against standard strains; the tested extract showed more potent inhibitory effects on both Gram (+) bacteria and Gram (-) bacteria. Thus, it could be concluded that due the presence of antioxidant components, the plant extract has well prospective for the management of diabetes and the related condition of oxidative stress. This knowledge will help find more potent antidiabetic principles from the natural resources for the clinical development of antidiabetic therapeutics.

Introduction

Diabetes mellitus (DM) is the collective name of metabolic abnormalities, primarily caused by the defect in secretion of insulin hormone by the pancreatic islets. It is chiefly manifested in the form of elevated levels of blood glucose (hyperglycemia). The reduced action of insulin on target tissues leads to a group of abnormalities, affecting the biochemistry and physiology of carbohydrate, fat, and protein.[1,2] According to the International Diabetes Foundation reports, the estimated value of diabetes among adults will reach 53% (592 million) by the year 2035. The DM stands 5th among the diseases that can lead to death around the world.[3] Certainly, a large number of synthetic drugs have been discovered in the past, but these drugs were found to have side effects. Therefore, researchers focused on developing new drugs from natural sources that are safe without any side effects. One of the recent developments in the field of natural products is the exploration of potent plant species, such as polyherbal.

As mentioned earlier, DM has a close association with other metabolic abnormalities; one of the core abnormalities is oxidative stress. Biochemical studies have revealed an increased generation of reactive oxygen species (ROS) in the cells and tissues of diabetic patients.[3] To tackle the ROS, the presence of potent antioxidant in the body of a patient is necessary because an antioxidant has the capacity of retarding or completely inhibiting the oxidation of other substances. In this regard, DPPH radical

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scavenging assay is one of the popular antioxidant assays. Combining the several medicinal plant extract in one formulation often gives more promising pharmacological and therapeutic effect than using a single medicinal plant. A formulation containing several medicinal plants extract is known as PHF. Due to their effective medicinal and therapeutic properties, such formulations are used all over the world over; conventional antibiotics having various side effects. However still, various herbal therapies, toxicological studies, and *in vitro* evaluation have not been carried out.[4] Ethnopharmacological studies are important for documenting and protecting cultural and traditional knowledge associated with the medical use of biodiversity.[5] This paper presents a survey on medicinal plants used by locals residents of Thanjavur, Tamil Nadu, India. Ethnopharmacological data were compared with available scientific information on the medicinal plants' efficacy and toxicology used from specialized databases, such as PubMed and Science Direct. Hence, this study aimed to determine the *in vitro* antidiabetic, antioxidant, and antimicrobial activity of a novel PHF to prove its efficacy in the treatment of various ailments.

Materials and Methods

Collection of Botanical Species

The botanical species of *Azadirachta indica* (*Meliaceae*), T*rigonella foenum-graecum* (*Fabaceae*), *Canthium coromandelicum* (*Rubiaceae*), *Barringtonia acutangula* (*Lecythidaceae)* were collected from Thanjavur, Tamil Nadu, India. The collected specimens are authenticated by Dr.S.John Brito, The Director, The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's college (campus), Trichy, Tamil nadu, India.

Preparation of Polyherbal Formulation

Polyherbal formulation (PHF) which consists dried plant powders of *Azadirachta indica*, T*rigonella foenum-graecum*, *Canthium coromandelicum* and *Barringtonia acutangula.* After collection, the plant's materials are washed and shade dried. After 15 days, the polyherbal were pulverized individually into a fine powder. Each plant powder was weighed accurately and mixed together in specific proportions. Finally, it was stored in an airtight container and used for further analysis. The composition of PHF was mentioned in Table 1.

Table 1: Composition of PHF

S.No	*Botanical name*	*Parts used*	*Each 100 gm Contains (gm)*
1	*Azadirachta indica*	Leaves	25.0
2	*Trigonella foenum-graecum*	Seeds	25.0
3	Canthium coromandelicum	Leaves	25.0
4	Barringtonia acutangula	Bark	25.0

Plant Extraction

Pulverized sample of PHF (100 g) was extracted with methanol (1:5 w/v) using mechanical shaker for 72 h. The extract was filtered using Whatman filter paper 1 and concentrated to yield crude extract.

Phytochemicals Screening

The phytochemicals screening was conducted on the crude extract following the method described by Odebiyi and Sofowara.[6] The following phytochemicals were screened for; saponins, phenols, flavonoids, alkaloids and terpenoids

GC-MS Analysis

This investigation was carried out to determine the possible chemical components of PHF by GC-MS.– QP 2010 PLUS SHIMADZU JAPAN. A methanol extract of PHF is used for GCMS analysis.

In vitro Antidiabetic Activity

The air-dried and coarse PHF powdered sample (10 g) was extracted with 100 mL methanol by maceration on an orbital shaker with agitation for five days at room temperature. The extract was filtered, and the residue was again extracted with same solvent for another five days and filtered. The filtered extracts were combined and concentrated using a rotary evaporator under reduced pressure at approximately 40°C and lyophilized to obtain the powdered extract. The powdered extract were analysed for their α-glucosidase and α-amylase inhibition assays. Percentage inhibition of the enzymes α-amylase and α-glucosidase were carried out by a previously optimized procedure.[7]

In Vitro α-Amylase Inhibitory Assay

A starch solution (1% w/v) was prepared by stirring 1 g starch in 100 mL of 20 mM of phosphate buffer (pH 6.9) containing 6.7 mM of sodium chloride. The enzyme solution was prepared by mixing 27.5 mg of porcine pancreatic amylase α-amylase (PPA) in 100 mL of 20 mM of phosphate buffer (PBS, pH 6.9) containing 6.7mM of sodium chloride. To 100 µL of (10, 20, 30, 40, 50 µg/mL) PHF extract, 200 µL porcine pancreatic amylase was added and the mixture was incubated at 37°C for 20 minutes. 100 µL (1%) starch solution was added and incubated at 37°C for 10 minutes to the reaction mixture. The reaction was stopped by adding 200 µL DNSA (1g of 3,5 dinitro salicylic acid, 30g of sodium potassium tartrate, and 20 mL of 2N sodium hydroxide was added and made up to a final volume of 100 mL with distilled water) and kept it in a boiling water bath for 5 minutes. The reaction mixture was diluted with 2.2 mL of water, and absorbance was read at 540 nm. For each concentration, blank tubes were prepared by replacing the enzyme solution with 200 µL in distilled water. Control, representing 100% enzyme activity, was prepared similarly, without extract. The experiments were repeated thrice using the same protocol.[8]

In Vitro α -Glucosidase Inhibition Assay

The inhibition of α-glucosidase activity was determined using the modified published method.[9] One mg of α-glucosidase was dissolved in 100 mL of phosphate buffer (pH 6.8). To 100 µL of (10, 20, 30,40, 50 µg/mL) PHF extract, 200 µL α-glucosidase were added, and the mixture was incubated at 37°C for 20 minutes. To the reaction mixture 100 µL 3mM *p*-nitrophenyl–D glucopyranoside (p-NPG) was added and incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 2 mL Na_2CO_3 0.1 M and the α-glucosidase activity was determined spectrophotometrically at 405 nm on spectrophotometer UV-VIS (Shimadzu UV-1800) by measuring the quantity of *p*-nitrophenol released from p-NPG.

Acarbose was used as a positive control of α-amylase and α-glucosidase inhibitor. The concentration of the extract required to inhibit 50% of α-amylase, and α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

The PHF extract concentration required to scavenge 50% of the radicals (IC_{50}) was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated by I % = (Ac-As)/Ac X 100, where Ac is the absorbance of the control and As is the absorbance of the sample.

Assay of Antimicrobial Activity

A total of 25 g of PHF extract was weighed and macerated in methanol, in the ratio of 1:4 (w/v). They were kept at room temperature for 24 hours, stirred every hours using sterile glass rod. Then it was filtered through the Whatmann No. 1 filter paper. Bacteria causing UTI were used in the present study. They were both gram-positive and gram negative. Such as *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus* and *Bacillus subtilis* were used. They were collected from the microbial type culture collection (MTCC) at Chandigarh, India. Pure cultures of bacterial pathogens in nutrient agar slant was transferred to tryptone broth and incubated at 37°C for 24 hours. The 6mm (diameter) discs were prepared from Whatmann No. 1 filter paper. The discs were sterilized by autoclave at 121°C. After the sterilization, the moisture discs were dried on hot air oven at 50°C.Then discs were mixed with chemical compounds separately and control discs were prepared. Antibacterial activity test was carried out following the modification of the method originally described by Bauer *et al.*10 Muller Hinton agar was prepared and autoclaved at 15 lbs pressure for 20 minutes and cooled to 45°C. The cooling media was poured on to sterile petri plates and allowed for solidification. The plates with media were seeded with the respective microbial suspension using sterile swab. The ethanol extract soaked discs were placed on each petri plate and also placed control and standard (Nitrofurantoin) discs. The plates were incubated at 37°C for 24 hours. After the incubation period, the diameter of the zone formed around the paper disc were measured and expressed in mm.

DPPH Scavenging Activity

The DPPH scavenging activity of the extract was determined by the method of Jain and Agarwal, (2008).[10,11] Various of concentrations of PHF extract (10 µL, 20 µL, 30 µL, 40 µL and 50 µL)) of test solution and 50 µL of DPPH (0.659 mM) solution are incubated at 25°C for 20 and the absorbance was read at 510 nm using shimadzu UV 1800 spectrophotometer, same procedure used for control without sample. Ascorbic acid served as the positive control. The % inhibition was calculated according to the following equation % Inhibition = $(A_0 - At)/A_0 \times 100$. Where A_0 was the absorbance of the control (blank, without extract) and At was the absorbance in the presence of the extract.

Data Analysis

The values are represented as Mean ± SEM of triplicates. Microsoft office excel was used to calculate IC_{50} value.

Results

Organoleptic Evaluation

The organoleptic characters such as appearance, color, odor, taste and texture of the formulation were carried out. The results are shown in Table 2.

GC-MS Analysis

The chromatogram of methanol extract of PHF was presented in Fig. 1. The relative retention time, as well as mass spectra of components, were compared with those of standard from NIST library. Totally 17 compounds were identified from PHF (Table 3). The identified compounds were esters, phytol, polyunsaturated omega-6 fatty acid (Linolenic acid), saturated fatty acids (Palmitic acid and myristic acid) etc.,

In vitro Antidiabetic Activity

The percentage inhibition of α-amylase and α-glucosidase exhibited by the methanol extract of PHF are depicted in Table. IC_{50} values are compared with standard drug acarbose and listed in Table 4.

In the current study, the inhibitory effect of PHF extract on carbohydrate hydrolyzing enzymes α-amylase and α-glucosidase were investigated. These enzyme inhibitors

Table 2: Organoleptic characteristics of PHF

S.No	*Parameters*	*In-house formulation*
1	Appearance	Powder
2	Colour	Green
3	Taste	Bitter
4	Odour	No Characteristic
5	Texture	Moderately coarse powder

Table 3: Components identified in the methanol extract of PHF by GCMS

Peak#	*R.Time*	*Area*	*Area (%)*	*Height*	*Height (%)*	*A/H*	*Name*
1	5.034	34023548	7.78	19137370	31.88	1.78	BENZOIC ACID, 2-HYDROXY-, METHYL ESTER
2	5.920	1911859	0.44	784474	1.31	2.44	Benzoic acid, 2-hydroxy-, ethyl ester
3	10.070	325355151	74.44	32671714	54.42	9.96	1,2-Benzenedicarboxylic acid, diethyl ester
4	10.633	42410562	9.70	2588173	4.31	16.39	.alpha.-D-Galactopyranoside, methyl
5	11.084	20341338	4.65	1604853	2.67	12.67	1,2-BENZENEDICARBOXYLIC ACID, DIETHYL ESTER
6	11.525	1817702	0.42	261164	0.44	6.96	4-BROMO-N-[(6-METHYL-2-PYRIDYL)
7	11.640	1210387	0.28	221907	0.37	5.45	1,2-Benzenedicarboxylic acid, dibutyl ester
8	11.785	944570	0.22	184766	0.31	5.11	Tetradecanoic acid (CAS) Myristic acid
9	12.173	176070	0.04	65114	0.11	2.70	1,2-Benzenedicarboxylic acid, diethyl ester
10	13.119	181514	0.04	69385	0.12	2.62	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
11	13.964	1977527	0.45	588545	0.98	3.36	Hexadecanoic acid (CAS) Palmitic acid
12	14.142	114280	0.03	28207	0.05	4.05	1,2-Benzenedicarboxylic acid, dibutyl ester
13	14.251	221111	0.05	104884	0.17	2.11	Pentadecanoic acid, ethyl ester
14	15.406	714216	0.16	278550	0.46	2.56	1,2-Benzenedicarboxylic acid, dioctyl ester
15	15.545	1448010	0.33	517950	0.86	2.80	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R--[R*,R*-(E)]]-(CAS) Phytol
16	15.829	1913668	0.44	314141	0.52	6.09	9,12-Octadecadienoic acid (Z,Z)- (CAS) Linol
17	15.966	814000	0.19	250273	0.42	3.25	2-ethylhexyl 2-ethylbutyrate
18	16.061	783410	0.18	192425	0.32	4.07	9,12-Octadecadienoic acid (Z,Z)- (CAS) Linol
19	16.158	175532	0.04	70681	0.12	2.48	11,14,17-Eicosatrienoic acid, methyl ester
20	22.885	513973	0.12	96441	0.16	5.33	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl)ester
		437048428	100.00	60031017	100.00		

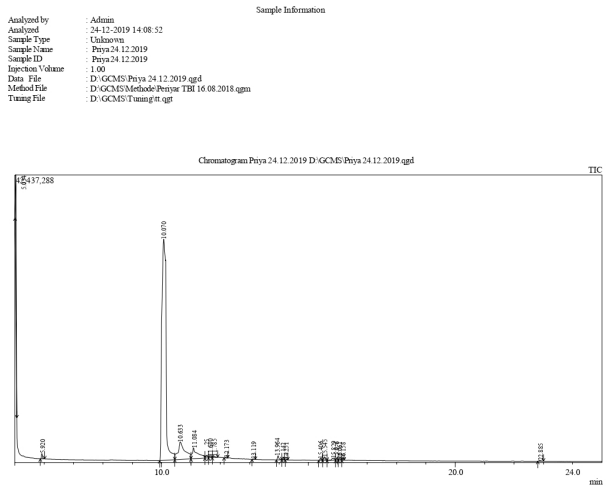

Fig. 1: GCMS chromatogram of methanol extract of PHF

antagonize these enzymes' activity and delay the digestion of carbohydrates, which prevents the sudden rise in blood glucose level, especially after meal.[12] Therefore, inhibition of these two enzymes is an attractive approach for the management of diabetes. In the present study, PHF inhibits α-amylase 73.03 ± 0.09%, and acarbose inhibits 75.51 ± 0.03% (Fig. 2). PHF inhibits glucosidase 58.28 ± 0.56% compared with standard drug acarbose, which inhibits 80.72 ± 0.08% (Fig. 3). This indicates that the methanolic

Table 4: IC_{50} values of standard drug acarbose and methanol extract of PHF

	IC_{50}(μg/mL)	
Sample	*α-amylase*	*α-glucosidase*
PHF extract	31.52 ± 0.74	53.13 ± 0.97
Acarbose	15.95 ± 0.24	13.54 ± 0.45

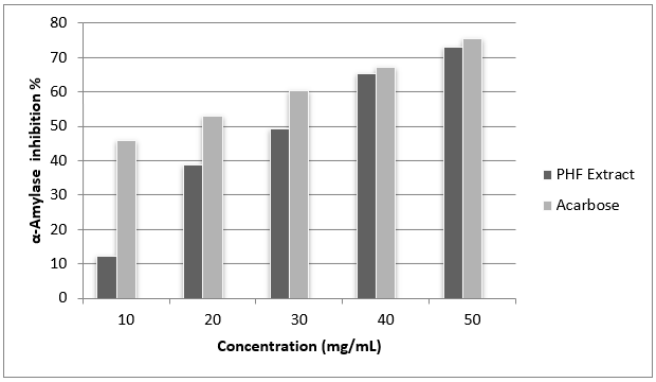

Fig. 2: The α- Amylase inhibitory activity of PHF extract and standard drug Acarbose

extract of PHF is very potent α-amylase and α-glucosidase inhibitor compared with acarbose. This could be justified that the nature of some extract constituents (phenols, flavonoids, steroids, alkaloids, terpenoids) present in the extract could be responsible for being effective inhibitors of α-amylase and α-glucosidase.[7]

Table 5: Assay of Antibacterial activity

S. No.	Bacteria Name	Zone of Inhibition (mm in diameter)		
		Control	Standard*	Sample
1	Escherichia coli	-	20	18
2	Klebsiella pneumonia	-	16	17
3	Staphylococcus aureus	-	25	17
4	Bacillus subtilis	-	35	32

*NIT-Nitrofurantoin (300µg).

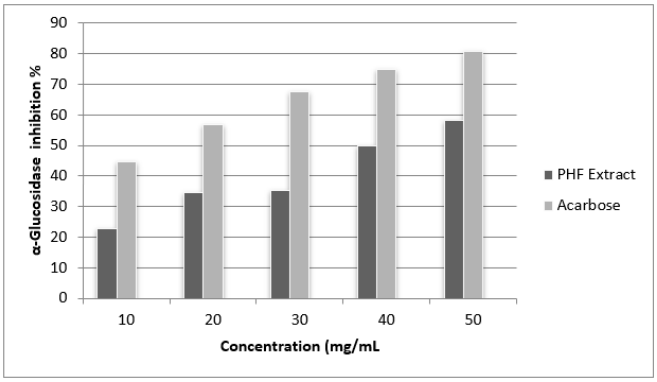

Fig. 3: The α-Glucosidase inhibitory activity of PHF extract and standard drug acarbose.

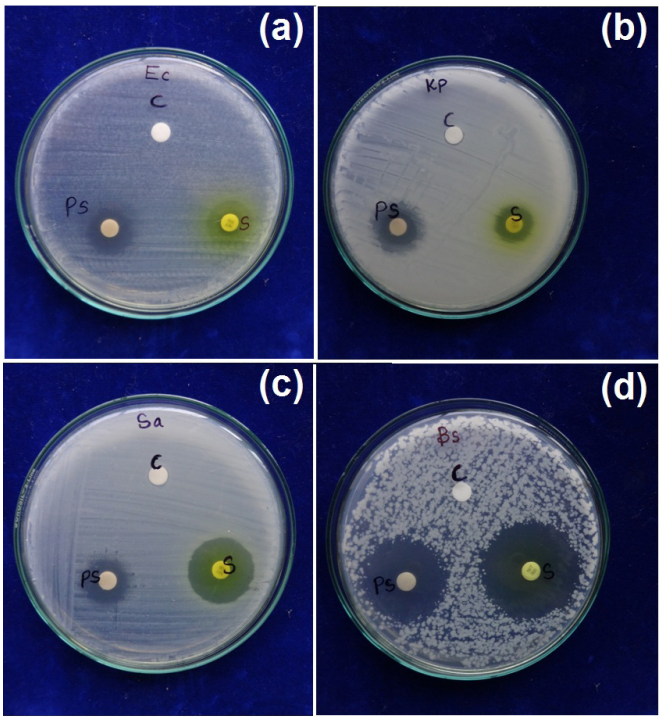

Fig. 4: Antimicrobial activity of PHF extract against test pathogens **(a)** *Escherichia coli* **(b)** *Klebsiella pneumonia* **(c)** *Staphylococcus aureus* **(d)** *Bacillus subtilis*

The bacterial activity of PHF extract *was* tested against gram (+) and gram (-) bacteria. The results are presented in Table 5 and Fig. 4. These results were also compared with a standard antibacterial drug (Nitrofurantoin). From the obtained results, PHF extract showed good antibacterial activity.

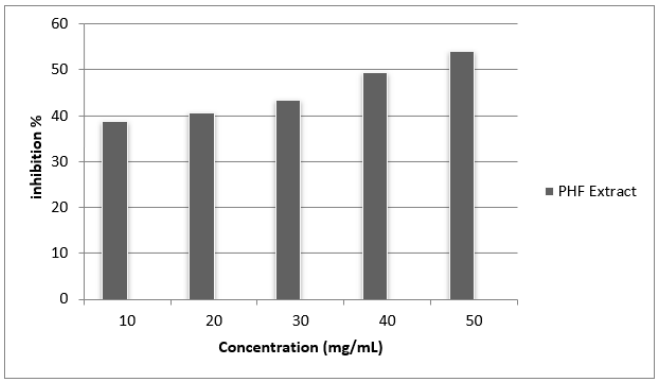

Fig. 5: DPPH radical scavenging activity of PHF extract

DPPH Scavenging Activity

The inhibition concentration at 50% inhibition (IC_{50}) was the parameter used to compare the radical scavenging activity. DPPH radical scavenging activity in a concentration-dependent manner (Fig. 5) and the IC_{50} was found to be 4.18 µg/mL. However, the activity was less when compared with the standard ascorbic acid (IC_{50} value 0.210 µg/mL). A lower IC_{50} meant better radical scavenging activity. The PHF extract showed a good scavenging activity on DPPH radical.

Discussion

Medicinal plant usage in ameliorating metabolic diseases is gaining more favor in research due to its phytochemical constituents.[13] In this present study, PHF is known to possess saponins, phenols, flavonoids, terpenoids, alkaloids. PHF showed good antibacterial activity against tested Gram-positive and Gram-negative bacteria. If antimicrobial therapy is indicated for UTI, it is important to determine the correct drug, dose, and therapy duration. Sometimes, as with acute uncomplicated cystitis, the clinical presentation is suggestive of a predominant organism (*E. coli*) with predictable antimicrobial susceptibility, and narrow-spectrum agents are appropriate for empiric treatment. Analyzing antibiotic susceptibility pattern of uropathogens help to overcome the therapeutic difficulties created by the rising antimicrobial-resistant bacteria and guides in choosing appropriate antibiotics.[14] Hence, we aimed at evaluating the pathogens causing UTI and study their antibiogram. The present study showed high antibacterial activity against the gram-positive *B. subtilis and* gram-negative *E.coli*, which are pathogens widely associated with urinary tract infections. Alpha-glucosidase is pivotal in increasing plasma glucose after consuming carbohydrate-rich meal therefore, inhibiting the action of α-glucosidase is therapeutic in managing diabetes complications. In this study, PHF attenuated α-glucosidase activity. This activity could be linked to its alkaloids and terpenoids components. In previous studies, alkaloids and terpenoids were reported to inhibit α-glucosidase activity and α-amylase.[15,16] The conventional treatments for diabetes

include reducing the demand for insulin, stimulation of endogenous insulin secretion, enhancement of the action of insulin at the target tissues, and the inhibition of the degradation of oligo disaccharides.[7,17,18] The inhibitors of α-glucosidase represent one group of drugs introduced in the management of T2D. The enzymes summarized as α-glucosidase are responsible for the breakdown of oligo- and/or disaccharides to monosaccharides. The inhibitory action of these enzymes leads to a decrease of blood glucose level, because the monosaccharides are the form of carbohydrates which are absorbed through the mucosal border in the small intestine. Another effective method to control diabetes is to inhibit the activity of an α-amylase enzyme responsible for the collapse of starch to more simple sugars (dextrin, maltotriose, maltose and glucose).[19] This is contributed by α-amylase inhibitors, which delay the glucose absorption rate, thereby maintaining the serum blood glucose in hyperglycemic individuals.[20]

Some inhibitors currently in clinical use are acarbose and miglitol which inhibit glycosidases such as α-glucosidase and α-amylase while others such as and voglibose inhibit α-glucosidase. However, many of these synthetic hypoglycemic agents have their limitations, are non-specific, produce serious side effects, and fail to elevate diabetic complications. These inhibitors' main side effects are gastrointestinal viz., bloating, abdominal discomfort, diarrhea, and flatulence.[21] Recently herbal medicines are getting more important in treating diabetes as they are free from side effects and less expensive compared to synthetic hypoglycemic agents.[22,23] From the previous study, the phytochemical constituents like saponin, phenols, flavonoids etc. studied in various plants such as *Proteus vulgaris, Euphorbia hirta, Cassia glauca* showed potential α-amylase inhibitors.[24] In pharmacological options, phytochemicals can act against T2DM, MS, and associated complications.[25] Extracts of *A.indica* and their components, especially alkaloids, have been documented for their potential activity against T2DM in various *in vitro* studies.[26,27] The role of medicinal plants in disease prevention is attributed to its antioxidant properties due to bioactive constituents' presence.[28] Thus, in this study, the antioxidant and antidiabetic activities of the methanol extract of PHF was carried out and a relationship between antioxidant and antidiabetic activities was established. Further works, however, still need to be carried out on the dose-response relationship and mechanisms of actions of all the effect observed so far.

Conclusion

In the present study, a novel PHF was prepared, biological activities of this novel polyherbal formulation have been analyzed. This study revealed that the PHF extract showed highest antibacterial effect on gram-positive and gram-negative bacteria. Also, we found that the extract of PHF have free radical scavenging activity and inhibitory activity against α-amylase and α-glucosidase, and this therapeutic potentiality could be exploited in the management of postprandial hyperglycemia in the treatment of T2DM. Although the effects of PHF extract have been established *in vitro,* these results indicate that PHF has potential as a crude drug and a dietary health supplement. The PHF showed significant enzyme inhibitory activity, so the compound isolation, purification, and characterization responsible for inhibiting activity have to be done to use antidiabetic agent. Further studies are also required to elucidate whether the plant has antidiabetic potential by in vivo to corroborate the plant's traditional claim. The combined use of ethnopharmacological survey and the scientific literature to select medicinal plants for phytotherapy programs benefited from the use of information from residents and the scientific community. This approach seeks to enhance the involvement of the community and health professionals in the local phytotherapy program.

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