

In vitro Inhibitory Effect of Polyherbal Formulation on Alpha-Amylase

Dr.S.M Gopinath¹, Priyanka.P², K.S.Dayananda³, Jagan Mohan Reddy⁴, Ashwini patil G.M⁵ Drishya V.Nair⁶

Professor, Department of Biotechnology, Acharya Institute of Technology, Bangalore, Karnataka, India¹

P.G Student, Department of Biotechnology, Acharya Institute of Technology, Bangalore, Karnataka, India²

Professor, Department of Biotechnology, Acharya Institute of Technology, Bangalore, Karnataka, India³

Professor, Department of Biotechnology, Acharya Institute of Technology, Bangalore, Karnataka, India⁴

Research student, Department of Biotechnology, Acharya Institute of Technology, Bangalore, Karnataka, India⁵

Student, B.Tech in Biotechnology, Vellore Institute of Technology, Vellore, Tamilnadu, India⁶

Abstract: Diabetes Mellitus is a metabolic disorder characterized by high blood sugar level caused due to deficiency of insulin secretion or insulin action. One of the therapeutic approach to treat Type II Diabetes is to lower the postprandial blood glucose level by inhibition of carbohydrate hydrolyzing enzyme such as alpha-amylase. In present investigation polyherbal formulation (PHF) composed of 22 medicinal plants having anti-diabetic property were selected from WHO monographs and evaluated for in vitro alpha-amylase inhibitory activity. Air dried powders of 22 medicinal plants were divided into four categories. One formulation named as (PHF1) was used and prepared by mixing the powders in an optimized ratio of 80:10:5:5. Extracts of PHF1 prepared by soxhlet method using polar and non-polar solvent was subjected to Inhibition assay by using Dinitro salicylic acid (DNS) method and phytochemical constituents in the extract was analyzed qualitatively as well as by GC-MS. The results revealed the presence of glycosides, steroids, terpenoids, saponins, phenols and tannins. In vitro study indicates that PHF1 Hot water extract showed maximum percentage inhibition of alpha-amylase activity. This hot water extract of PHF1 can be effective in lowering postprandial hyperglycemia (PPHG).

Keywords: Type II Diabetes, Postprandial hyperglycemia (PPHG), Polyherbal formulation (PHF), Alpha-Amylase inhibition, Acarbose.

I. INTRODUCTION

Diabetes Mellitus (DM) is a chronic metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [1]. There are many therapeutic strategies for the treatment of Type II Diabetes mellitus. Available therapies for treating type II diabetes include stimulation of endogenous insulin secretion, enhancement of action of insulin at target tissues and the inhibition of alpha-amylase enzyme to decrease the degradation of starch to reducing sugar. The drugs commonly used in clinic to handle or control diabetes are insulin, sulfonylureas, biguanide, glycosidase inhibitors, aldose reductase inhibitor, thiazolidinediones, carbamoylmethyl benzoic acid, insulin-like growth factor [2]. Antidyslipoproteinemic agents as gemfibrozil and flavastatin are used [3]. The α -amylase (α -1,4-glucan-4-glucanohydrolases; E.C. 3.2.1.1) is one of the major secretory products of the pancreas and salivary glands, playing a role in digestion of starch and glycogen and can be found in microorganisms, plants and higher organisms [4]. Alpha-Amylase enzyme catalyses the initial step in hydrolysis of starch to mixture of oligosaccharides consisting of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units that contain both α -1,4 and α -1,6 linkages. These are further degraded to glucose by Alpha-Glycosidase

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

which on absorption enters bloodstream. Rapid degradation of starch by Alpha-Amylase enzyme leads to elevated post-prandial hyperglycemia (PPHG). Thus decreasing the degradation of starch to reducing sugar by inhibition of Alpha-Amylase enzyme plays a key role in control of diabetes. Inhibitors of pancreatic Alpha-Amylase prevent starch breakdown and absorption there by lowering post prandial glucose levels and also weight loss in humans [5, 6]. Acarbose and miglitol which inhibit Alpha-Amylase and Alpha-Glucosidase are currently in clinical use. These synthetic drugs not only lower glucose level but also induce side effects and fail to treat diabetic complications. The main side effects of these synthetic drugs are abdominal distension, bloating, flatulence and diarrhea [7]. It has been suggested that such adverse effects might be caused by excessive inhibition of pancreatic Alpha-Amylase resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon [8]. Therefore herbal medicines are gaining more importance in the treatment of diabetes as they are free from side effects and also less expensive when compared with synthetic drugs [9]. In India Ayurveda and other Indian traditional medicines have used plants for treatment of many diseases. Ethnobotanical studies of traditional herbal remedies used to treat diabetes have identified more than 1,200 species of plants with hypoglycemic property [10]. WHO (World Health Organization) has recommended the evaluation and medicinal properties of plants effective in such systems [11]. Screening natural sources such as medicinal plants can lead to potent and specific inhibitors for Alpha-Amylase therefore natural Alpha-Amylase inhibitors from the dietary plants can be used as effective therapy for post prandial hyperglycemia [12]. Number of medicinal plants and their formulations are used for treating Diabetes in the traditional Indian Ayurvedic system. Major formulations used in Ayurveda are based on herbs used as decoctions, infusion, tinctures and powders. Drug formulation in Ayurveda is based on two principles: (a). Use as single drug, and (b). Use of more than two drugs. When two or more herbs are used in formulation they are known as polyherbal formulation. It is evident that there are many herbal formulations of varying potency since these preparations act by different mechanism. Thus selected medicinal plants were subjected to solvent extraction and the resulting extracts were evaluated for their porcine pancreatic Alpha- Amylase inhibitory potential, screened for the presence of amylase inhibitors under invitro conditions by performing GC-MS and the extracts were subjected to phytochemical analysis in order to identify the probable inhibitory phytoconstituents.

II. MATERIAL AND METHODS

Chemicals such as soluble starch, isopropanol, methyl-butyl-tertiary ether, petroleum ether, chloroform, and ethanol were of Analytical grade. PPA (Porcine pancreatic alpha amylase), starch and Acarbose was purchased from Sigma Aldrich USA.

Plant material

Anti-diabetic medicinal plants that are used were selected from WHO monographs. Powders used are of high quality and proved to have anti-diabetic property. Out of twenty two medicinal plant powders fifteen powders were purchased from nearby local market and remaining seven medicinal plant material (fenugreek, *Tinospora cardifolia*, curry leaves, stevia, onion, garlic and ginger) were air dried for 25 days after complete drying each plant material were finely crushed, powdered and stored in brown bottle. Plant material used are shown in table 1.

Table1: Plant material used for the study

Plant species	Family	Part used
<i>Syzygium cumini</i>	Myrtaceae	Seed
<i>Phyllanthus emblica</i>	Phyllanthaceae	Seed
<i>Trigonella Foenum-graceum</i>	Fabaceae.	Seed
<i>Momordica charantia</i>	Cucurbitaceae	Seed

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

Gymnema sylvestre	Asclepiadaceae	Leaves
Tinospora cordifolia	Menispermaceae	Leaves
Murraya koenigii	Rutaceae	Leaves
Azadirachta indica	Meliaceae	Leaves
Moringa oleifera	Moringaceae	Seed
Stevia	Asteraceae	Leaves
Curcuma longa	Zingiberaceae	Rhizomes
Ocimum tenuiflorum	Lamiaceae	Leaves
<i>Piper nigrum</i>	Piperaceae	Seeds
Withania somnifera	Solanaceae	Root
Cuminum cyminum	Apiaceae	Seeds
Coriandrum sativum	Apiaceae	Seeds
YAM	Dioscoreaceae	Tuber
Macrotyloma uniflorum	Fabaceae	Seeds
Glycine max	Fabaceae	Beans
Allium cepa	Amaryllidaceae	Bulb
Allium sativum	Amaryllidaceae	Rhizome
<i>Zingiber officinale</i>	Zingiberaceae	Rhizome

Preparation of polyherbal formulation

Medicinal Powders were first divided into four categories as powders having major antidiabetic property, medium antidiabetic, hepatoprotective and anticholesteremic property. One polyherbal formulations (PHF1) was used and prepared by mixing the powders of above said categories in the ratio 80:10:5:5. Details of formulation is given in Table 2 . Air dried plant material was crushed, powdered and bioactive compounds from powders were extracted using soxhlet apparatus with polar and non polar solvent. The solvent used were hot water, cold water, ethanol, isopropanol, petroleum ether, methyl-butyl-tertiary ether and chloroform. Cold water extracts (PHF1) was obtained by adding distilled water to the crushed material in ratio 1:4 and kept in rotary shaker for 24h at 30 degree Celsius and at 130 rpm respectively. After 24 h it was filtered and the resulting extract was stored in refrigerator. Hot water extracts were obtained by soxhlet extraction were 400ml of distilled water to 100 grams of plant powders (PHF1) .Similarly bioactive compounds from medicinal powders were extracted using organic solvent in ratio 1:3 by soxhlet extraction. Crude extracts obtained were transferred to glass bottles and stored in refrigerator for further studies.

Table2: Polyherbal formulation PHF1 (80:10:5:5)

Major antidiabetic	Medium anti-diabetic	Anti Hepatoprotective	Anti-Hypertensive
Jambul	Curry leaves	Turmeric	Horse gram
Amla	Neem	Tulsi	Soybean
Fenugreek	Drum stick	Pepper	Onion
Bitter guard	Stevia	Jeera	Garlic

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

Gymnema sylvestre		Dhania	Ginger
Tinospora cordifolia			Ashwagandha
			YAM

Invitro Porcine Pancreatic Alpha-Amylase inhibition assay

DNS (3, 5 Dinitrosalicylic acid) assay: The determination of α -Amylase inhibition was carried out by quantifying the reducing sugar (glucose equivalent) liberated under the assay condition. The enzyme inhibitory activity was expressed as decrease in units of glucose liberated [15]. Plant extract concentrations ranging from 10-100 μ g were incubated with 1ml of 1unit PPA Enzyme for 30minutes at 37°C. After incubation 1ml of 1% buffered starch was added and the mixture was further incubated for 10minutes at room temperature. The reaction was stopped by adding 1ml DNS reagent and the contents were heated in boiling water bath for 5minutes. Blank was prepared without plant extract and enzyme which was replaced with equal quantity of 0.1M phosphate buffer. Control representing 100% enzyme activity without plant extract was also included. The absorbance was read at 540nm using UV Spectrophotometer. The reducing sugar released from starch was estimated as glucose equivalent from standard graph. Standard antidiabetic drug Acarbose was used as positive control. The antidiabetic property was determined through inhibition of alpha amylase which was expressed as percentage of inhibition and calculated by following equation:

% Inhibition = Absorbance of control – Absorbance of test/Absorbance of control *100

IC50 values of Acarbose and PHF1 extracts of different solvents were determined from plots of percentage inhibition versus concentration (μ g/ml). IC50 value is defined as the concentration of extract required to inhibit 50% of PPA (Porcine Pancreatic Alpha-Amylase) activity.

Qualitative phytochemical analysis:

Extracts were tested for the presence of alkaloids, tannins, flavonoids, polyphenols, saponins etc using standard procedures. [16]. 1:10 dilution of extracts was prepared and then subjected to phytochemical screening.

GC-MS Analysis:

PHF1 Hot water extract which showed maximum inhibition (88.23%) on PPA activity was subjected to GCMS equipped with Rtx-5 capillary column (30m x 0.25mm ID). Helium was the carrier gas with flow rate of 1ml/min, the injector mode – split ratio 5.0, the injection volume 1 μ l, the temperature program used is as follows: 150°C then increased to 300°C held at 300°C for 15.00 min. The identification of components was based on the comparison of their mass spectra with those of NIST-WILEY Library.

III.RESULTS

In the present study Polyherbal formulation(PHF1) extracts of seven different organic solvents exhibited significant reduction in alpha-amylase activity. Acarbose standard antidiabetic drug which was used as positive control at concentrations 10-100 μ g/ml showed PPA inhibitory activity from 18.75% to 58.60% with an IC50 value of 83.34 μ g/ml [Table 4, figure1].PHF1 hot water extract at concentration10-100 μ g/ml showed maximum inhibitory effects on Alpha-Amylase activity from 23.5% to 88.23% with an IC50 value of 40 μ g/ml and 28 μ g/ml respectively [Table5, figure 2]. PHF1 Ethanolic, cold water, Isopropanol, Petroleum ether, Chloroform and Methyl tertiary butyl ether extracts at concentrations 10-100 μ g/ml showed moderate inhibitory effects on Alpha-Amylase activity from 17.6% to 70%, 26.4% to 61.76%, 29.4% to 55.8%, 41.17% to 57.9%, 18.2% to 52.9% and 11.7% to 40% respectively with an IC50 value of 72 μ g/ml,70 μ g/ml,74 μ g/ml,60 μ g/ml,90 μ g/ml and 96 μ g/ml[Table 6, figure 4 to 9]. Qualitative phytochemical screening revealed presence of Glycosides, steroids, Triterpenoids, phenols, tannins and saponins [Table 8]. Results pertaining to GC-MS analysis are given in [Table 9].

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

Table 4: IC50 Values of Acarbose on Alpha-Amylase inhibition

Standard drug	Concentration($\mu\text{g/ml}$)	% Inhibition	IC50 Value
	10	18.75	83.33 $\mu\text{g/ml}$
	20	22.41	
Acarbose	40	29.73	
	60	38.74	
	80	48.27	
	100	58.60	

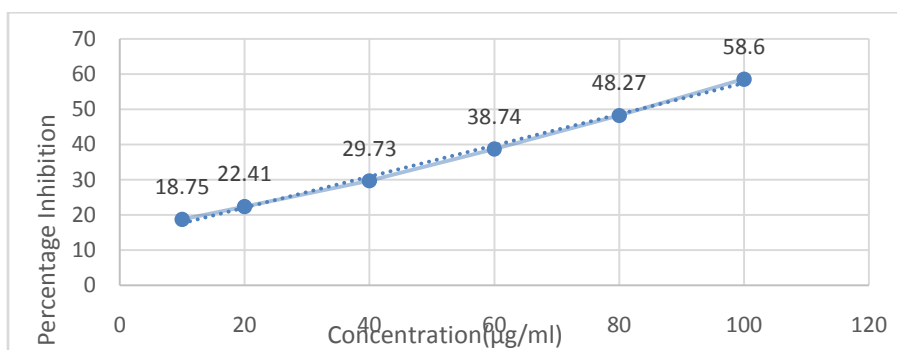


Figure 1: Acarbose percentage inhibition graph

Table 5: PHF1 showing maximum inhibitory effects on alpha-amylase activity

Polyherbal formulation	Extract	Concentration($\mu\text{g/ml}$)	% Inhibition	IC50 Value
PHF1	Hot water	10	23.5	40 $\mu\text{g/ml}$
		20	41.1	
		40	50	
		60	61.7	
		80	73.5	
		100	88.23	

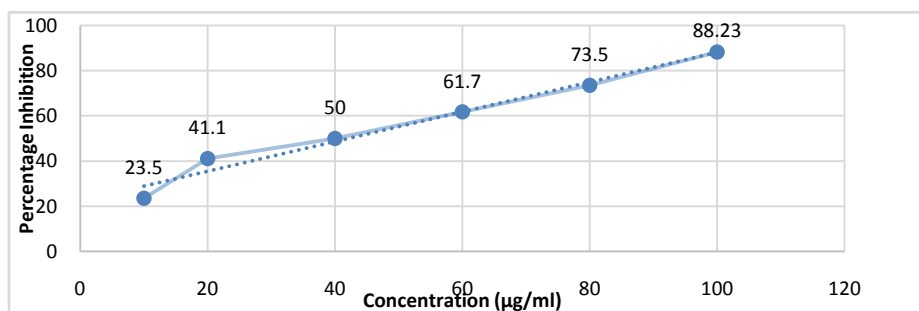


Table 6: PHF 1 showing moderate inhibitory effects on alpha-amylase activity

Polyherbal formulation	Extract	Concentration($\mu\text{g/ml}$)	% Inhibition	IC50 Value
PHF1	Ethanol	10	17.6	72 $\mu\text{g/ml}$
		20	26	
		40	32.3	

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

		60	41	
		80	61	
		100	70	
PHF1	Cold water	10	26.4	70µg/ml
		20	35.2	
		40	38.2	
		60	44.1	
		80	50.1	
		100	61.76	
PHF1	Isopropanol	10	29.4	74µg/ml
		20	35.2	
		40	41.1	
		60	47	
		80	52.9	
		100	55.8	
PHF1	Petroleum ether	10	41.17	60µg/ml
		20	42.9	
		40	47.05	
		60	49.4	
		80	52.9	
		100	57.9	
PHF1	Chloroform	10	18.2	90µg/ml
		20	26.4	
		40	29.4	
		60	38.2	
		80	44.1	
		100	52.9	
PHF1	Methyl tertiary Butyl ether	10	11.7	96µg/ml
		20	17.6	
		40	26.4	
		60	32.3	
		80	34.1	
		100	40	

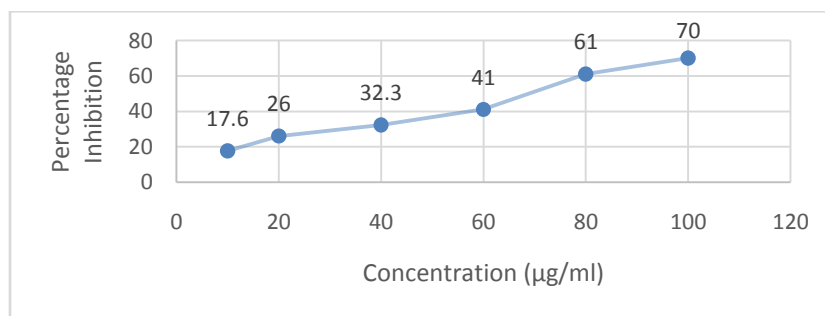


Figure 4: PHF1 Ethanol extract percentage inhibition graph

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

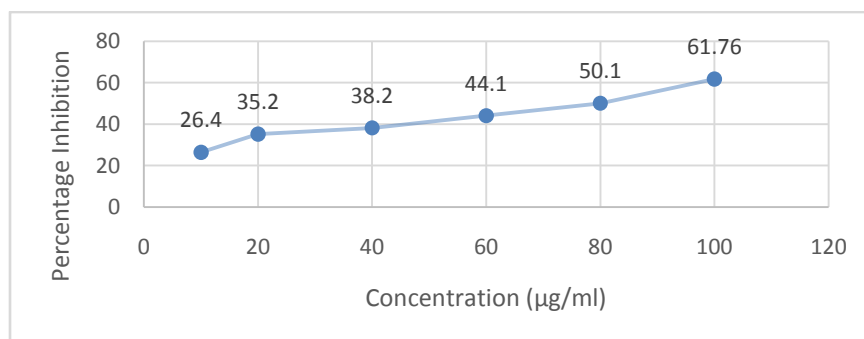


Figure 5: PHF1 Cold water extract percentage inhibition graph

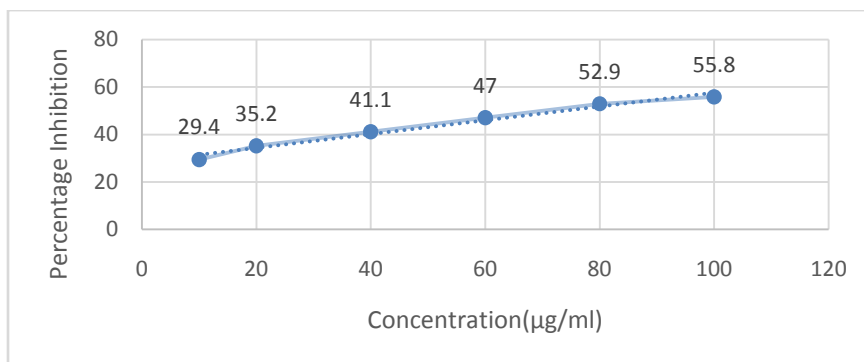


Figure 6: PHF1 Isopropanol extract percentage inhibition graph

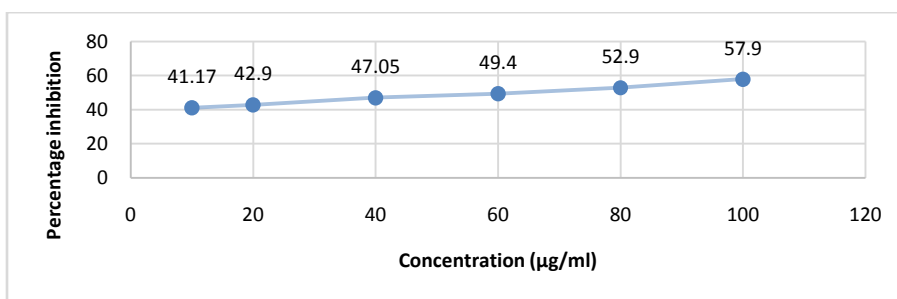


Figure 7: PHF1 Petroleum ether extract percentage inhibition graph

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

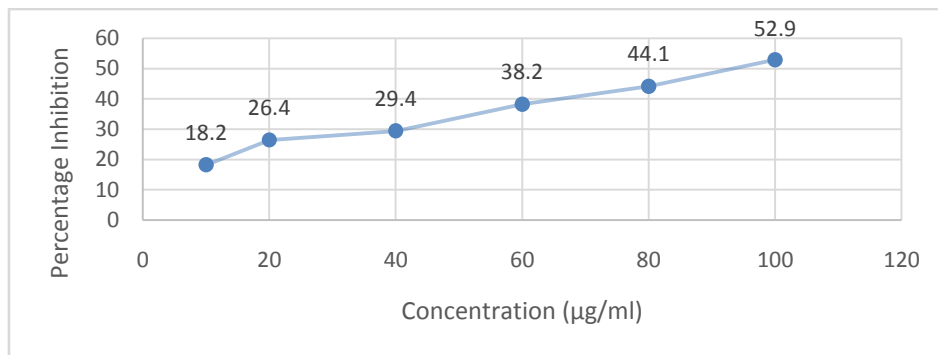


Figure 8: PHF1 Chloroform extract percentage inhibition graph

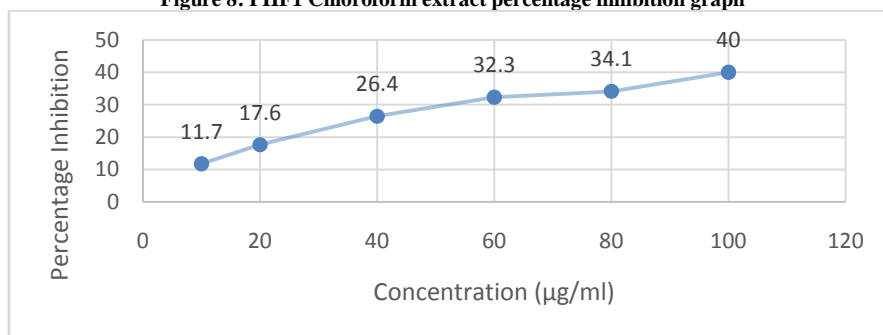


Figure 9: PHF1 Methyl tertiary butyl ether percentage inhibition graph

Table 8: Results of Qualitative phytochemical analysis of PHF1

Test	Ethanol extract	Hot water extract	Cold water extract	Isopropanol extract	Chloroform extract	Petroleum ether extract	Methyl tertiary butyl ether extract
Carbohydrate	+	+	+	+	+	+	+
Glycosides	-	+	+	+	+	+	+
Alkaloids	-	+	-	-	-	-	-
Steroids	-	-	+	+	+	+	+
Triterpenoids	+	+	-	-	-	-	-
Flavonoid	-	-	-	-	-	-	-
Phenol	+	+	+	+	+	+	+
Tannins	+(G)	+(G)	-	-	-	-	-
Saponins	+	-	-	-	-	-	+

EOH-

Ethanol extract, HW- Hot water, CW-Cold water, PE-Petroleum ether

MBTE- Methyl butyl tertiary ether

G- Indicates green colour

+ Indicates presence

- Indicates absence

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

Table 9: Compounds detected in PHF1 Hot water extract

Sl no	Retention time	Name of Compound	Molecular formula	Molecular weight	Peak area%
1.	2.494	2-Furancarboxaldehyde, 5-hydroxymethyl	C ₆ H ₆ O ₃	126	3.66
2.	3.538	1,2,3-Benzenetriol	C ₆ H ₆ O ₃	126	1.79
3.	4.628	d-Gluco-heptulosan	C ₇ H ₁₄ O ₇	210	3.76
4.	7.085	Ar-tumerone	C ₁₅ H ₂₀ O	216	0.91
5.	11.887	Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	8.23
6.	14.518	Phytol	C ₂₀ H ₄₀ O	296	1.03
7.	15.097	9,12-Octadecadienoic acid(Z,Z)-Linoleic acid	C ₁₈ H ₃₂ O ₂	280	51.86
8.	15.416	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	284	11.36
9.	17.683	Cis-6-Shogaol	C ₁₇ H ₂₄ O ₃	276	0.59
10.	17.764	Ethyl linoleolate	C ₂₀ H ₃₆ O ₂	308	1.03
11.	20.326	1H-Benzocyclohepten-7-ol,2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl	C ₁₅ H ₂₆ O	222	1.23
12.	23.764	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	C ₁₉ H ₃₄ O ₂	294	1.91
13.	25.681	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl	C ₃₀ H ₅₀	410	1.87
14.	26.965	Piperidine,1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]	C ₁₇ H ₁₉ NO ₃	285	2.11
15.	29.268	1-Eicosanol, n-Eicosanol	C ₂₀ H ₄₂ O	298	1.36
16.	29.856	Alpha-Tocopherol-beta-D-mannoside	C ₃₅ H ₆₀ O ₇	592	0.63
17.	31.729	Stigmasterol	C ₂₉ H ₄₈ O	412	1.50
18.	32.642	Gamma-Sitosterol	C ₂₉ H ₅₀ O	414	4.05
19.	34.141	Lupeol	C ₃₀ H ₅₀ O	426	1.11

IV.DISCUSSION

In this study PHF1 was evaluated for their respective alpha-amylase inhibitory activity. The result indicates that PHF1 hot water extract showed maximum inhibitory effect on Alpha-Amylase with an IC50 value 40µg/ml and 28µg/ml which is less than Acarbose suggesting that PHF1 Hot water extract could be a promising lead extract. This is due to the presence of phytochemicals that acts as potential alpha-amylase inhibitors such as Glycosides, steroids, phenols, triterpenoids etc. To analyze which phytochemical compound in PHF1 Hot water extract was responsible to inhibit the enzyme alpha-amylase, sample was subjected to GC-MS. It was noted that in PHF1 Hot water extract, 19 major compounds were detected which is shown in [Table 9]. Thus due to existence of Hexadecanoic acid, 9,12-Octadecadienoic acid(Z,Z)-Linoleic acid, lupeol, Phytol and other compounds PHF1 Hot water extract showed maximum inhibitory activity on Alpha-Amylase activity. It is well known that certain flavonoids and phytol exhibit hypoglycemic activity and is also known for their ability of beta-cell regeneration of pancreas [17, 18]. Sterols have also shown to decrease blood sugar in experimental animal models [19]. Thus the significant anti-diabetic effect of the extract may be due to the presence of flavonoids, Triterpene, steroids, palmitic acid ester etc. The activities of some phytochemicals with compound nature of flavonoids, palmitic acid (hexadecanoic acid ethyl ester and nhexadecanoic acid), unsaturated fatty acid and linolenic acid (docosatetraenoic acid and octadecatrienoic acid) as antimicrobial, anti-inflammatory, antioxidant hypocholesterlemic, cancer preventive, hepatoprotective, antiarthritic, antihistimic, antieczimic and anticoronary [20]. Oral administrations of Grewiaum bellifera extract at 250mg and 500 mg / kg body wt for 28days showed decrease in fasting blood when compared with that of

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 9, September 2013

standard drug Glibenclamide due to the presence of Triterpene, Asarone, Diterpene, linoleic acid ester, Flavonoid compound, Steroid compound which was analyzed in GC-MS [21]. Isopropanol extract of *Ocimum tenuiflorum*, *Morus Alba* and *Linum usitatissimum* showed greater than 50% PPA inhibitory activity, potent alpha-amylase inhibitor responsible for this inhibition was identified by GC-MS analysis which indicated that Camphene in *Ocimum tenuiflorum*, Hexadecanoic acid and 9, 12-Octadecadienic acid in *Morus Alba* and 2-Cyclopentene-1-undecanoic acid in *Linum usitatissimum* were the major compounds responsible for maximum inhibitory activity [22]. *Syzygium aromaticum*-derived oleanolic acid (OA) inhibits alpha-amylase and alpha-glycosidase enzyme with an IC₅₀ value of 62.11 μg/ml less when compared with IC₅₀ value of Acarbose 80.25 μg/ml, thereby decreasing the absorption of glucose across the small intestine [23]. Ethanol extract of *Allium sativum* and *Allium cepa* has been reported for alpha-amylase inhibitory effects with an IC₅₀ value of 17.95 and 16.36 mg/ml [24]. Ethanol extract of *Mangifera indica*, *Azadirachta indica* and petroleum ether extract of *Murraya koenigii* showed maximum percentage inhibition on alpha-amylase activity with IC₅₀ value of 37.86 μg/ml, 62.99 μg/ml and 59 μg/ml [25]. *Acalypha indica* is used in Indian traditional medicinal system and it is reported that chloroform and hexane extracts to possess alpha-amylase activity [26]. Omega-3-fatty acids have been found to be essential for normal growth and development and may play an important role in the prevention and treatment of coronary artery disease, hypertension, diabetes, and arthritis, other inflammatory and auto immune disorders and cancer [27]. The present study indicates that hot water extracts of Polyherbal formulation (PHF1) composed of twenty medicinal plants can be useful in management of postprandial hyperglycemia because the hot water extract possess important bioactive compounds which acts as alpha-amylase inhibitors, thus lowering PPHG (Post prandial hyperglycemia) level in type II Diabetic patients.

V. CONCLUSION

Indian Ayurvedic system from ancient times has used the medicinal plants to treat diabetes. PHF used in our study is composed of a plant which has hypoglycemic property; formulations were tested for anti-diabetic property by subjecting the extracts of PHF to Alpha-Amylase inhibition assays. One of the mechanism to control blood sugar level in type II Diabetic patients is to lower the PPHG level. This can be targeted by reducing the activity of Alpha-Amylase enzyme which is responsible for conversion of starch to reducing sugar. Thus the Results obtained suggests that PHF1 Hot water extract is said to have more than five bioactive compounds which inhibits the activity of alpha-amylase enzyme therapy can be used as an Oral hypoglycemic agent to control PPHG level.

Future Study: Antioxidant property of PHF1 can be evaluated as well as in vivo studies need to be performed to confirm antidiabetic and antioxidant property of PHF.

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Vol. 2, Issue 9, September 2013

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