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Pawan Kumar
Department of Toxicology, Frontier Lifeline Hospital and Dr. K. M. Cherian Heart Foundation, Mogappair, Chennai – 600101, India.

Patchaiappan Pravinkumar
Department of Toxicology, Frontier Lifeline Hospital and Dr. K. M. Cherian Heart Foundation, Mogappair, Chennai – 600101, India.

Shabeer Sakkeena Iqbal
Department of Clinical Biochemistry, Frontier Lifeline Hospital and Dr. K. M. Cherian Heart Foundation, Mogappair, Chennai – 600101, India.

Kalathil Sadasivan Pillai
Department of Toxicology, Frontier Lifeline Hospital and Dr. K. M. Cherian Heart Foundation, Mogappair, Chennai – 600101, India.

Mathews Michael
Department of Toxicology, Frontier Lifeline Hospital and Dr. K. M. Cherian Heart Foundation, Mogappair, Chennai – 600101, India.

Correspondence:
Mathews Michael
Department of Toxicology, Frontier Lifeline Hospital and Dr. K. M. Cherian Heart Foundation, Mogappair, Chennai – 600101, India.

Phytochemical profile, heavy metals contents and antioxidant activities of an antidiabetic polyherbal formulation

Pawan Kumar, Patchaiappan Pravinkumar, Shabeer Sakkeena Iqbal, Kalathil Sadasivan Pillai, Mathews Michael

Abstract
The aim of this study was to investigate the phytochemical profile, heavy metal contents and antioxidant activities of an antidiabetic polyherbal formulation (PHF) containing *Azadirachta indica* A. Juss, *Commiphora mukul*, *Curcuma longa* L, *Emblica officinalis* Gaertn, *Garcinia cambogia* Gaertn, *Gymnema sylvestre* Retz, *Momordica charantia* L, *Ocimum sanctum* L and *Trigonella foenum-graecum* L. Total phenolic content, flavonoids and flavonols present in the formulation were determined by standard methods. Antioxidant potential was evaluated using total antioxidant activity, ferric reducing antioxidant power and 2, 2-diphenylpicrylhydrazyl (DPPH) assays. The chemical composition of the formulation was studied using Gas Chromatography–Mass Spectrometry (GC-MS). Heavy metals were analysed using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). The total phenolic content of the PHF was 232.2 mg gallic acid equivalent/g. Total flavonols and total flavonoid contents of the PHF were 180.7 and 135.3 mg quercetin equivalent/g, respectively. The PHF showed a reducing power of 26.8 mg ascorbic acid equivalent/g. Total antioxidant capacity was found to be 144.4 mg ascorbic acid equivalent/g. The ferric reducing antioxidant power (FRAP) of the PHF was 1260.9 (μM Fe (II)/g). The DPPH radical scavenging activity expressed as IC_{50} value was 2.6 μg/mL for gallic acid and 3.1 μg/mL for the formulation. The levels of heavy metals, such as arsenic, cadmium, chromium, lead and mercury in the PHF were within the World Health Organization (WHO) limits. Out of the eight bioactive compounds identified in the formulation, the major compounds were 10-Octadecenoic acid methyl ester, Octadecanoic acid methyl ester and Hexadecanoic acid methyl ester. The results show that the polyherbal formulation possesses good antioxidant activity and could be a potential candidate for antidiabetic studies.

Keywords: Polyherbal formulation, Gas Chromatography- Mass Spectrometry, Phytochemicals profile, bioactive compound, Antioxidant activity.

1. Introduction
Diabetes mellitus is a hyperglycemic metabolic disorder characterized by defective insulin production and peripheral insulin resistance or both. Hyperglycemia is known to produce reactive oxygen species (ROS) which play a central role in the complications of diabetes [1] such as microvascular and macrovascular complications [2]. As per the International Diabetes Federation, 382 million people worldwide were affected with diabetes mellitus disease in 2013 by 2035 this will rise to 592 million [3]. Management of diabetes mellitus is considered a global problem and successful treatment with affordable cost and no/or minimum side effects is yet to be discovered. Therefore, it has become the need of the hour to search for new drugs and interventions to manage this metabolic disorder.

World Health Organization supports the use of traditional medicines provided they are proven to be efficacious and safe [4]. Plants, in all aspects of life, have served a valuable starting material for drug development [5]. Plants are sources of bioactive compounds and minerals through the primary and secondary metabolic processes. The most important bioactive constituents of plants are phenols, steroids, terpenoids, carotenoids, flavanoids, alkaloids, tannins and glycosides. These bioactive constituents may play an important role in protecting cells and organs from oxidative damage.

Heavy metals contamination is one of the major problems with plant extract or products. They are usually present in the plant formulations in extremely low concentrations, making their quantitative analysis a challenging task. In the present study, inductively coupled plasma optical emission spectrometry (ICP-OES) was used for the determination of trace metals because of its speed and wide availability.

The ingestion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing [6, 7]. The present study was carried out to find out the phytochemical profile, heavy metal contents and antioxidant activities of an antidiabetic polyherbal formulation (PHF). The PHF was prepared using a patented aqueous extraction procedure. The plants used in the preparation of the formulations were *Azadirachta indica* A. Juss, *Commiphora mukul*, *Curcuma longa* L, *Emblica officinalis* Gaertn, *Garcinia cambogia* Gaertn, *Gymnema sylvestre* Retz, *Momordica charantia* L, *Ocimum sanctum* L and *Trigonella foenum-graecum* L. These plants were selected based on a literature survey and prevailing traditional practices in combating diabetes and associated complications.

A. indica belongs to the family Meliaceae commonly known as neem tree. Almost every part of the neem tree has been used to treat a number of human ailments. The aqueous extract of neem leaf showed a fall in blood glucose levels in diabetic rats [8]. Treatments with petroleum ether extracts of kernel and husk of neem alone significantly protected against oxidative stress in heart and erythrocytes in streptozocin-diabetic animals [9]. *C. mukul* is commonly known as guggul tree belongs to the Burseraceae family. The active ingredients of this plant have excellent cholesterol lowering property [10]. *C. longa* commonly known as turmeric belongs to the family Zingiberaceae. Curcumin and its derivatives extracted from the rhizomes of this plant have been reported to possess hypoglycemic effects on genetically diabetic KK-Ay mice [11]. Orally ingested curcumin reversed obesity-associated inflammation and diabetes in mice [12]. *E. officinalis* commonly known as Indian gooseberry belongs to the family Euphorbiaceae. *E. officinalis* significantly reduced cholesterolemia and aortic sudanophilia in rabbits [13]. *G. cambogia* belongs to the family Clusiaceae / Guttiferae and is commonly known as gamboge. Hydroxycitric acid, an active ingredient extracted from *G. cambogia* has been reported to be an effective inhibitor of fatty acid synthesis in rat liver [14]. *G. sylvestre* belongs to the family Asclepiadaceae and commonly known as 'periploca of the woods'. Gymnemic acid extracted from *G. sylvestre* is reported to increase faecal excretion of cholesterol and cholic acid-derived bile acids in rats [15] and stimulate insulin release *in vitro* [16]. *M. charantia* belongs to the family Cucurbitaceae and commonly known as bitter gourd. Aqueous extract of *M. charantia* has been shown to partially stimulate insulin release from isolated beta cell of obese-hyperglycaemic mice [17]. *O. sanctum* belongs to the family Lamiaceae and commonly known as Holy basil. A controlled clinical trial conducted in 40 patients with type 2 diabetes indicated the positive effect of *O. sanctum* in controlling both fasting and postprandial glucose levels without any adverse effects [18]. *T. foenum-graecum* belongs to the family Fabaceae/Papilionaceae and commonly known as fenugreek. The administration of fenugreek seed powder to diabetic rats revealed to lower the blood glucose level and partially refurbish the activities of key enzymes of carbohydrates and lipid metabolism to near normal levels [19].

The formulations prepared of herbal extracts using organic solvents, usually are contaminated with the solvents. Therefore, the PHF used in the present study was prepared using a patented aqueous extraction procedure [20]. The present study was aimed to evaluate the phytochemical profile, heavy metals contents and antioxidant activities of the PHF.

2. Materials and Methods
2.1 Plant Materials
The Polyherbal formulation was gifted by M/s. Lanson Biotech, Chennai. The list of plants used for the preparation of the PHF and the content of individual plant in the formulation are given in Table 1.

Table 1: Ingredients of Polyherbal formulation

S. No.	Botanical Name	English Name	% Used	Part Used
1.	*Azadirachta indica* A. Juss.	Neem	10	Seed kernel
2.	*Commiphora mukul*	Indian Bdellium	1	Resin – guggulsterone
3.	*Curcuma longa* L.	Turmeric	10	Rhizomes
4.	*Emblica officinalis* Gaertn.	Indian Gooseberry	20	Fruit pulp
5.	*Garcinia cambogia* Gaertn.	Gamboge	1	Fruit pulp
6.	*Gymnema sylvestre* Retz.	Periploca of the woods	20	Leaves
7.	*Momordica charantia* L.	Bitter gourd	20	Fruit pulp with seeds
8.	*Ocimum sanctum* L.	Holy Basil	8	Leaves & seeds
9.	*Trigonella foenum-graecum* L.	Fenugreek	10	Seeds

2.2 Extraction of the plant parts for the preparation of the PHF
The herbs were collected from organic farms, which were free from pesticide residue. Then the herbs were cleaned in natural mineral water. Required quantities of fruit pulp with seeds of *Momordica charantia* L, fruit pulp of *Emblica officinalis* Gaertn and the leaves of *Gymnema sylvestre* Retz were extracted by pounding without any application of heat at room temperature and fresh juice was obtained. The outer coating of *Azadirachta indica* (neem) seed kernal were removed and was extracted using rotary press, without any external heating. The seeds of *Trigonella foenum-graecum* L, rhizome of *Curcuma longa* L, fruits of *Garcinia cambogia* Gaertn and resins of *Commiphora mukul*, Leaves & seeds of *Ocimum sanctum* L were air dried at room temperature and made a fine powder using a mixer-grinder. The mixture of fine powder obtained was soaked in the juice obtained from the other plants, which acted as a natural organic solvent. The extract was then dried at room temperature without any heating process.

2.3 Chemicals and reagents
The chemicals used in this study were ascorbic acid (Qualigens fine), quercetin (Sigma Aldrich), $AlCl_3$ (Fisher Scientific), Acetic acid (Fisher Scientific), 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4'-disulfonic acid (Sigma-Aldrich), ferrous chloride (Qualigens fine), trichloroacetic acid, gallic acid, Folin-Ciocalteu reagent, sodium carbonate, potassium ferricyanide, phosphate buffer, sulfanilic acid and all other chemicals used were of analytical grade (Himedia).

2.4 GC-MS (gas chromatography-mass spectrometry) analysis

GC-MS analysis of the PHF was performed using Agilent 6890N GC system, coupled to a JEOL GC Mate II mass spectrometer fused with a HP-5MS (5% phenyl-95% methylsiloxane). For GC-MS detection, an electron ionization system was operated in the electron impact mode with ionization energy of 70 eV. Pure helium gas was used as the carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 1 uL was employed splitless. The PHF was dissolved in ethanol prior to injection. The injector temperature was maintained at 220 °C, the ion chamber temperature was 250 °C, the oven temperature was programmed from 50 °C to 250 °C at 10 °C/min. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 50 to 600 Da. The solvent delay was 0 to 2 min, and the total GC-MS running time was 40 minutes. The spectrum of compounds obtained through GC – MS were compared with the National Institute Standard and Technology (NIST) library to identify individual components. The relative percentage amount of each component was calculated by comparing its average peak area to the total peak area.

2.4.1 Identification of phytocomponents

Interpretation of mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) which is having more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were determined. The concentrations of the identified compounds were expressed in term of peak area percentage. The percentage of peak area is proportional to amount of the compound present in the formulation.

2.5 Quantitative estimations

All the estimations were performed using aqueous solution of the sample with a concentration of 1 mg/mL.

2.5.1 Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent method of Singleton et al [21]. The reaction mixture was prepared by mixing 0.5 mL of sample solution, 2.5 mL of 10% Folin-Ciocalteu reagent dissolved in water and 2.5 mL of 7.5% of Na_2CO_3. Blank was concurrently prepared using 0.5 mL distilled water instead of PHF solution. The samples were thereafter incubated in an incubator at 45°C for 45 min. The absorbance was read on a spectrophotometer (Shimadzu UV-1800) at 765 nm. The phenolic content was calculated as Gallic acid equivalent from the calibration curve of Gallic acid standard solutions (range: 20–100 μg/mL). The phenolic content was expressed in terms of mg gallic acid equivalent (mg of GAE/g of the PHF).

2.5.2 Determination of total flavonoids content

The total flavonoids were determined using $AlCl_3$ method of Ordonez et al [22]. A volume of 0.5 mL of 2% $AlCl_3$ aqueous solution was added to 0.5 mL of sample. The contents were mixed well and incubated for 1 hour at room temperature for yellow colour appearance; the absorbance was read on a spectrophotometer (Shimadzu UV-1800) at 420 nm. Concurrently, the same procedure was used for the standard solutions of quercetin (concentrations: 20-100 μg/mL). Total flavonoids content was calculated using the calibration curve

and expressed as mg quercetin equivalent (mg of QUE/g of the PHF).

2.5.3 Determination of total flavonols content

The total flavonols content were determined using the $AlCl_3$ method of Kumaran and Karunakaran [23]. One millilitre of the PHF solution was mixed with 1.0 mL of 2% $AlCl_3$ prepared in distilled water and 1.5 mL of 5% sodium acetate solution was added. The mixture was incubated at 20 °C for 2.5 hours and the absorption was read at 440 nm on a spectrophotometer (Shimadzu UV-1800). Concurrently, the same procedure was used for the standard solutions of quercetin (concentrations: 20 - 100 μg/mL) for calibration curve. Total flavonols contents were calculated as mg quercetin equivalent (mg QUE/g of the PHF).

2.6 Antioxidant Potential

2.6.1 Total antioxidant capacity

The total antioxidant activity of the PHF was evaluated by the phosphomolybdenum method of Prieto et al [24]. An aliquot (0.1 mL) of the sample solution was mixed with 1.0 mL of reagent solution (0.6 M Sulphuric acid, 28 mM Sodium phosphate, and 4.0 mM Ammonium molybdate). The mixture was covered and incubated at 95 °C for 90 min. After cooling the mixture, the absorbance was read at 695 nm on a spectrophotometer (Shimadzu UV-1800) against blank. The blank solution contained 1.0 mL of reagent solution and 0.1 mL of distilled water, and it was incubated under the same conditions. Ascorbic acid was used as standard (concentrations: 20-100 μg/mL) for calibration curve. The antioxidant activity was expressed as mg ascorbic acid equivalent/g of PHF.

2.6.2 Reducing power assay

The reducing power capacity of the PHF was determined using the method described by Oyaizu [25]. An aliquot (200 μL) of the PHF solution was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. The mixture was incubated at 50 °C for 30 minutes. Thereafter, 2.5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged for 10 minutes at 4000 rpm. Finally, 2.5 mL of the upper layer was taken and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% aqueous $FeCl_3$ and the absorbance was recorded at 700 nm using a spectrophotometer (Shimadzu UV-1800). Ascorbic acid was used as reference standard. The result was expressed as mg ascorbic acid equivalent/g of PHF.

2.6.3 Ferric reducing antioxidant power assay

The ferric reducing activity was determined using the modified method of Benzie and Strain [26]. The stock solutions included, 300 mM acetate buffer (3.1 g $CH_3 COONa•3H_2O$ and 16 mL $CH_3 COOH$, pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3•6H_2O$. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3•6H_2O$ and incubated at 37 °C for 10 min. Two hundred microliter of $FeSO_4$ at different concentrations (20 - 100 μM) was allowed to react with 1800 μL of the working solution for 30 min in the dark condition. The same procedure was used for the comparative standard solution of ascorbic acid as well as the PHF. Readings of the colour product (ferrous tripyridyltriazine complex) were taken at 593 nm on a Spectrophotometer (Shimadzu UV-1800). FRAP (Ferric Reducing Antioxidant

Power) values were expressed in μM Fe (II)/g of PHF or ascorbic acid.

2.6.4 DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The DPPH radical scavenging assay of the PHF was measured in accordance with the procedure described by Blios [27]. The PHF solution (10-50 μL) was mixed with 0.5 mL of 0.1 mM methanolic solution of DPPH and incubated in dark for 30 minutes at room temperature. After incubation, the absorbance of each solution was measured at 517 nm using spectrophotometer (Shimadzu UV-1800). The same procedure was used for standard solution of gallic acid at different concentrations (1-5 μg/mL).

The scavenging ability of the plant extract was calculated using the equation:

$$\text{DPPH Scavenging activity (\%)} = \frac{\text{Abs}_{control} - \text{Abs}_{sample}}{\text{Abs}_{control}} \times 100$$

Where, $\text{Abs}_{control}$ is the absorbance of DPPH + methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract or standard.

The result of DPPH radical scavenging was expressed as IC_{50} calculated by using GraphPad software.

2.7 Heavy metals analysis using inductively coupled plasma optical emission spectrometry (ICP-OES)

The PHF sample (0.1 g) was digested with 3 mL of nitric acid in a CEM microwave digester using MARSX press (self-regulating microwave vessel) for 10 minutes under controlled conditions (i.e., Maximum power- 800W, % Power -75, Ramp - 10 min, Temperature – 200 ºC). The digested solution was made up to 25 mL using de-ionized water and it was thoroughly filtered using Whatman 40 filter paper and the clear solution was analyzed by inductively coupled plasma optical emission spectrometry (PERKIN ELMER OPTIMA 5300 DV ICP-OES). The operating conditions employed for ICP-OES determination were: RF power -1300 W; plasma flow - 15 L min-1; auxiliary flow - 0.2 L min-1; nebulizer flow - 0.8 L min-1; pump rate - 1.5 mL min-1. The ICP-OES analysis was carried out on axially viewed plasma. The emission intensities were obtained for the most sensitive lines free of spectral interference. The Individual metal concentration was obtained in mg/L. The results were also expressed as mg/kg of the PHF by using below mentioned formula.

$$\text{Sample concentration (mg/kg) dry-weight basis} = \frac{C \times V}{W}$$

Where,
C = Concentration of PHF (mg/L), V = Volume of solution prepared (L), W = Weight of sample used (kg).

3. Results and Discussion
The details of bioactive compounds revealed by GC-MS analysis of the PHF are provided in Table 2.

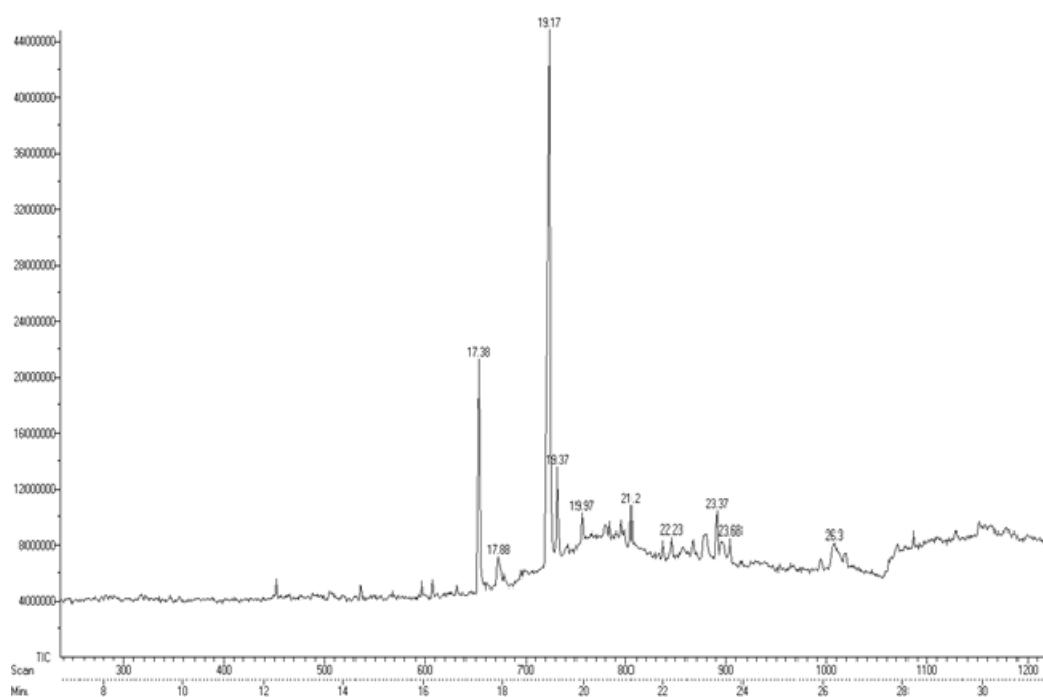**Table 2:** Bioactive compounds identified by GC-MS analysis

S. No	Bioactive compound	Molecular Formula	Molecular Weight	RT	Peak area %
A.	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.487	19.2	38.53
B.	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298.503	19.37	31.79
C.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.450	17.38	10.77
D.	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-methyl ester	$C_{21}H_{38}O_2$	322.525	19.97	7.18
E.	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354.610	23.37	5.08
F.	Dasycarpidan-1-methanol, acetate (ester)	$C_{20}H_{26}N_2O_2$	326.432	26.3	3.32
G.	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326.557	21.2	2.22
H.	1,2'-Benzedicarboxylic acid, butyl 2-methylpropyl ester	$C_{16}H_{22}O_4$	278.343	17.88	1.07

One of the major compounds found, 10-Octadecenoic acid, is a bio-converted form of oleic acid. Octadecanoic acid methyl ester, Hexadecanoic acid methyl ester, Eicosanoic acid methyl ester, Docosanoic acid methyl ester, Dasycarpidan-1-methnol acetate[ester], [1,1'-Bicyclopropyl]-2-octanoic acid 2'-hexyl-methyl ester and 1,2'-Benzedicarboxylic acid butyl 2-methylpropyl ester are commonly known as Stearic acid, Palmitic acid, Arachidonic acid, Behenic acid, AC1LB29U, Caprylic acid and Butyl isobutyl phthalate, respectively as mentioned in Table 3. Oleic acid, a monounsaturated fatty acid, is reported to have anti-diabetic and anti-inflammatory properties. Oleic acid increased insulin secretion in the glucose dependent insulin producing rat pancreatic cell line INS-1[28]. The inflammatory cytokine TNF-α level is reported to have increased in insulin resistance type 2 diabetes. It has been reported that oleic acid is capable of reversing the inhibitory effect of the inflammatory cytokine TNF-α [28]. Wu et al. reported that oleic acid increases the expression of PPARδ through a GPR40-PLC-calcium pathway, and the increase of PPARδ further regulates lipid metabolism and insulin sensitivity in steatotic status in HepG2 cells [29]. The reported therapeutic activities of various bioactive compounds identified in the PHF are given in Table 3.

Table 3: Therapeutic activity of the identified phytocompounds

S. No	Bioactive compounds	Other Name	Therapeutic activity
A.	10-Octadecenoic acid, methyl ester	Oleic acid	Increased insulin production in INS-1 cell and decreased TNF-α [28], Compensate insulin resistance in steatotic cells [29].
B.	Octadecanoic acid, methyl ester	Stearic acid	Activation of Stearoyl-CoA desaturase-1 (SCD1) [30].
C.	Hexadecanoic acid, methyl ester	Palmitic acid	Palmitate may be elongated to stearate[31], Activation of Stearoyl-CoA desaturase-1 (SCD1), Improved insulin resistance[32]
D.	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-methyl ester	Caprylic acid	No activity reported.
E.	Docosanoic acid, methyl ester	Behenic acid	Reduced body weight and visceral fat deposition[32]
F.	Dasycarpidan-1-methnol, acetate[ester]	AC1LB29U	No activity reported
G.	Eicosanoic acid, methyl ester	Arachidonic acid	No activity reported
H.	1,2'-Benzedicarboxylic acid, butyl 2-methylpropyl ester	Butyl isobutyl phthalate	Inhibit the action of α-glucosidase[33,34]

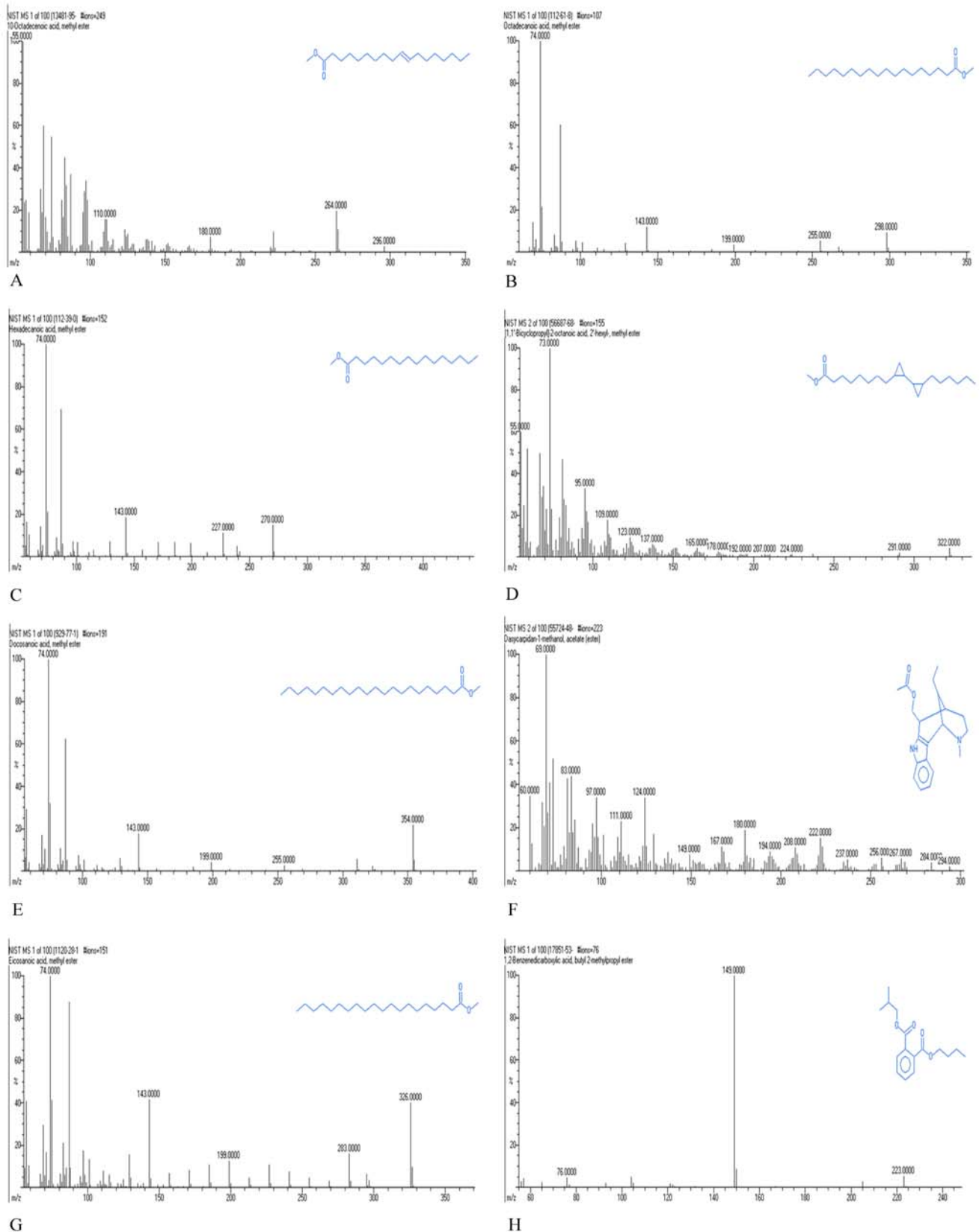**Fig 1:** GC-MS Chromatogram of the PHF

Fig 2: Mass spectrum and structure of phytocomponents identified by GC-MS of the PHF.

A. 10-Octadecenoic acid methyl ester, **B**. Octadecanoic acid methyl ester, **C**. Hexadecanoic acid methyl ester, **D**. [1, 1'-Bicyclopropyl]-2-octanoic acid 2'-hexyl-methyl ester, **E**. Docosanoic acid methyl ester, **F.** Dasycarpidan-1-methnol acetate[ester], **G.** Eicosanoic acid methyl ester, **H.** 1,2'-Benzedicarboxylic acid butyl 2-methylpropyl ester,

The total phenolic content of the formulation was 232.2 mg gallic acid equivalent/g of the PHF. The total flavonols and flavonoids contents of the PHF were 180.7 and 135.3 mg quercetin equivalent /g of the PHF, respectively (Table 4). Phenols, flavonols and flavonoids may play an important role in protecting cellular oxidative damage in many

chronic diseases such as diabetes, obesity, cardiovascular problem and ageing [35, 36] and also possess antioxidant properties [37]. Herbal drugs which contain polyphenol compounds like flavonoids are supposed to be responsible for the radical scavenging effect. Epidemiological studies have revealed that the consumption of flavonoids is effective in lowering the risk of coronary heart diseases [38].

Table 4: Result of quantitative estimation of the polyherbal formulation

Sample	Total phenolic content* (mg equiv. GAE/g)	Total flavonoid content # (mg equiv. QUE/ g)	Total flavonols content# (mg equiv. QUE/ g)
PHF	232.2± 0.69	135.3 ± 0.93	180.7 ± 1.72

Note:* = mg gallic acid equivalent/g of the PHF, # = mg quercetin equivalent/g of the PHF, Data are expressed as Mean ± SD (N=3).

The total antioxidant capacity was found to be 144.3 mg ascorbic acid equivalent/g of the PHF (Table 5). Polyphenols possess excellent antioxidant activity [24]. Reducing power of the formulation was 26.8 mg ascorbic acid equivalent/g of the PHF (Table 5). Increasing absorbance indicates an increase in the reductive ability of the sample. The ability of a compound to transfer electron is a significant indicator of its potential as an antioxidant [39]. The total ferric reducing antioxidant power (FRAP) of the formulation was 1260.9 μM Fe (II)/g of the PHF which was about 52% of the FRAP value of ascorbic acid. [2403.4 μM Fe (II)/g] (Table 5). Principally, FRAP assay treats the antioxidants in the sample as reductant in a redox linked colorimetric reaction. Antioxidant compounds which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus break the radical chain reaction [26].

The concentration dependent curve of DPPH radical scavenging activity of the formulation was compared with gallic acid. The highest percentage DPPH scavenging activity shown by the formulation was 52% whereas it was 83.3% for the standard gallic acid. The DPPH results are expressed as IC_{50} value. The calculated IC_{50} value was 2.58 μg/mL for gallic acid, whereas it was 3.13 μg/mL for the PHF formulation. This result suggests that the formulation contains compounds that are capable of donating hydrogen to a free radical in order to remove abnormal electron which is responsible for radical's reactivity.

Table 5: Antioxidant activity

S. No.	Sample	Total antioxidant© power (mg ascAE/g)	Reducing power© (mg ascAE/g)	FRAP value@ (μM Fe (II)/g)	DPPH IC_{50} (μg/mL)
1.	PHF	144.3 ± 2.01	26.8 ± 0.16	1260.9 ± 30.43	3.13
2.	Ascorbic acid	-	-	2403.4 ± 26.09	2.58

Note: © = mg ascorbic acid equivalent/g of the PHF, @ = FRAP value is expressed in μM Fe (II)/g of the PHF or ascorbic acid. The values are represented as mean ± SD (N=3).

The ICP-OES analysis showed that the values of arsenic, cadmium, chromium, lead and mercury were within the limits recommended by the WHO (Table 6).

Table 6: Heavy metal contents of PHF

S. No.	Parameter	Observed Value (mg/kg)	WHO permissible limit (PPM)[40]	Detectable Limit (mg/L)
1.	Arsenic	< BDL	5	0.053
2.	Cadmium	< BDL	0.3	0.002
3.	Chromium	2.0	2	0.007
4.	Lead	< BDL	10	0.042
5.	Mercury	< BDL	0.2	0.1

Note: BDL = below detectable limit

4. Conclusion
Eight bioactive compounds were detected in the polyherbal formulation. The major compounds were 10-Octadecenoic acid, Octadecanoic acid and Hexadecanoic acid. The values of heavy metals such as arsenic, cadmium, chromium, lead and mercury were found to be within the permissible limit in the PHF. Presence of phenols, flavonoids and flavonols indicated that the polyherbal formulation possess excellent antioxidant property. The PHF is a potential source of natural antioxidants and may be a potential candidate for the treatment of diabetes and diabetic complications. Further studies are needed to investigate the pharmacological profile of the formulation using *in vitro*, *in vivo* models.

5. Statistical Analysis
Experimental data were expressed as mean ± standard deviation (SD)

6. Conflict of Interest
None of the authors have any conflict of interest.

7. Acknowledgement

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