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HYPOGLYCEMIC AND ANTIPLATELET EFFECTS OF UNSAPONIFIED PETROLEUM ETHER FRACTION AND ISOLATED COMPOUNDS OF METHANOL EXTRACT OF CLERODENDRUM PHLOMIDIS LINN. F. LEAVES

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ABSTRACT

Methanol extract of the leaves of Clerodendrum phlomidis Linn. f. (Lamiaceae) has been reported for antidiabetic activity. The aim was to explore in detail the various fractions and isolated compounds from methanol extract of the leaves of C. phlomidis for hyopglycemic and antiplatelet effects. Residual fraction of methanol extract (RFME), unsaponified petroleum ether fraction of methanol extract (USPEF) and crude polyamine fraction (CPF) were studied by streptozotocin-nicotinamide (STZ-NAD) induced diabetic rat model (100 and 200 mg/kg BW, p.o. daily for 30 days). Metformin was used as standard drug along with diabetic and normal control. Compounds (CPI–VI) were isolated from bioactive USPEF. CPII and CPVI were studied at 15 and 30 mg/kg BW, p.o. daily for 30 days. All fractions, CPII and CPVI were also studied for protein tyrosine phosphatase 1B (PTP1B) inhibition and adenosine diphosphate (ADP) induced antiplatelet aggregation study. CPI (1-hexadecanol), CPII (clerosteryl palmitate), CPIII (β-carotene), CPIV (β-sitosterol), CPV (lupeol) and CPVI (clerosterol) were isolated from USPEF. CPII and CPVI showed moderate antidiabetic and antiplatelet effects. USPEF 200 mg/kg showed significant changes ($p<0.01$) in all parameters. USPEF also showed significant IC_{50} value of 23.50±1.1 and 11.12±0.8 µg/mL in PTP1B inhibition and ADP induced platelet aggregation study respectively. It was concluded that USPEF exhibits synergistic antidiabetic and antiplatelet activities.

KEY WORDS

Protein tyrosine phosphatase 1B, Platelet aggregation, Histopathological studies, Clerosterol, Clerosteryl palmitate.

1 INTRODUCTION:

Type 2 diabetes is a progressive disease characterized by insulin resistance in peripheral tissues and/or impaired insulin secretion by the pancreas. At molecular level, the mechanism of insulin resistance in type 2 diabetes involves post-receptor defects in insulin signalling[1]. The knowledge of heterogeneity of type 2 diabetes is increasing; hence there is a need to look for more efficacious agents for its treatment rather than managing hyperglycemia. Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator in both insulin and leptin signalling pathways[2]. In the insulin signalling pathway, PTP1B dephosphorylates the insulin receptor substrate IRS-1[3,4]. Overwhelming evidence suggests that inhibiting PTP1B represents a highly promising approach to treat diabetes[5].

Traditional herbal medicines are prescribed widely in developing countries because of their time-tested effectiveness and relatively low cost. An exhaustive study on medicinal plants may lead to isolation of interesting leads of novel chemical entities. Additionally, there exists a possibility of deriving new

knowledge on the mechanism of action of therapeutic agents which, in turn, may help in better understanding of the etiopathogenesis and the course of various diseases. The World Health Organisation (WHO) has recommended evaluation of the medicinal plants used for the treatment of diabetes. Therefore, it is prudent to look into medicinal plants as alternative resource for the treatment of diabetes.

Clerodendrum phlomidis Linn. f. (Lamiaceae) is an important and well known medicinal plant in Ayurveda and Siddha system of medicines. It is commonly known as Thalludhalai, Agnimantha or Arani and is a constituent of more than 50 indigenous drug formulations. Agnimantha is very essential medicinal plant mentioned in texts since vedic period. It is described as one of ten herbs of dashamula. The ayurvedic properties of *C. phlomidis* are: rasa - tikta, katu, kashaaya and madhura; guna - rooksha and laghu; veerya – ushna; vipaka – katu[6]. Due to its bitter and pungent nature *C. phlomidis* is considered to normalize the vitiated kapha and vata dosa[7]. *C. phlomidis* is used in different ayurvedic formulations such as Ayushyavardhaak tel, Bhratpanchamula, Chandraprabha vati, Lavanbhasker churna, Abhayarisht, Chavanprasha, Dasamularista, Ashwagandharishta, Mritasanjivani, Dasamula kvatha churna, Haritakiavleh, Indukanta ghrta, Dhanvantara ghrta, Gorocanadi vati, Narayana taila, Ras pitari, Vrahat panchamuli and Muthu marunthu (a siddha polyherbal formulation)[8]. Its popular uses include the treatment of inflammation and diabetic conditions[9]. Pectolinaringenin, scutellarein, clerodin, clerodendrin, clerosterol, 24β-ethylcholesta-5,22E,25-triene-3β-ol, lup-20(29)-en-3-triacontanoate, 4,2',4'-trihydroxy-6'-methoxychalcone-4, 4'α-D-diglucoside, 7-hydroxyflavone, 7-hydroxyflavanone-7-O-glucoside and α-L-rhamnopyranosyl-(1→2) α-D-glucopyranosyl-7-O-naringin-4'-O-α-D-glucopyranoside-5-methyl ether are reported earlier from *C. phlomidis* in various literatures[8]. Preliminary investigation has revealed the antidiabetic activity of methanol leaf extract of *C. phlomidis*[10]. Thus, it was thought to thoroughly investigate the various fractions of methanol leaf extract for *in vivo* and *in vitro* antidiabetic activity.

The American Heart Association (AHA) considers platelet hyperaggregability to be one of the important risk factors for cardiovascular disease in diabetic patients[11]. Hence, AHA and American Diabetes Association (ADA) recommend antiplatelet therapy as a primary prevention strategy in diabetic patients with increased cardiovascular risk[12]. Numerous epidemiological studies also support the early treatment of diabetic cardiovascular complications with antiplatelet agents[13]. In our current study, fractions and the compounds isolated from leaf methanol extract of *C. phlomidis* were also evaluated for platelet aggregation inhibitory activities.

2 SUBJECTS AND METHODS:

2.1 Materials

All reagents used were of technical or analytical grade and were purchased from Sigma-Aldrich, Spectrochem and Merck. All solvents were of analytical grade or HPLC grade and were distilled prior to use. The ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ or C_6D_6 using Bruker (Swiss) Avance II spectrometer (100 or 400 MHz). Chemical shifts are reported in δ units downfield from TMS as an internal standard. The CHNOS analyses were recorded using Thermo Finnigan Flash EA 1112 (Italy) analyser.

2.2 Plant material

Leaves of *C. phlomidis* were collected on October 2009 from out-fields of Trichy city, Tamil Nadu, India. The plant material was identified and authenticated by Dr. A. Rajan, Field Botanist, The Survey of Medicinal Plants and Collection Unit, Government Arts College, Ootacamund, Tamil Nadu, India. A voucher specimen (Pharmacy/HDT/CP/08-09/MKM/15) has been deposited in the Herbarium of Medicinal Plants, Pharmacy Department, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India.

2.3 Preparation of extract and fractions

Air-dried leaves (2000 g) of *C. phlomidis* were grounded and extracted with methanol in Soxhlet apparatus for 48 h. The extract was evaporated to dryness under reduced pressure on rotary evaporator (Rotavapor, Buchi) and further dried in desiccator to yield methanol extract. The methanol extract was further fractioned by centrifuging with 3 x 500 mL of petroleum ether (60-80 °C) at 1000 g for 15 min. The supernatants were combined, evaporated to dryness under reduced pressure on rotary evaporator and further dried in desiccator to yield petroleum ether fraction of methanol extract. The insoluble residue was designated as residual fraction of methanol extract (RFME). The petroleum ether fraction of methanol extract was

saponified as per the reported procedure[14] and the unsaponified matter was designated as unsaponified petroleum ether fraction of methanol extract (USPEF). A crude polyamine fraction (CPF) was prepared from 100 g of air-dried grounded leaves of *C. phlomidis* following the earlier described method[15].

2.4 Animals

Healthy adult Albino rats of Wistar strain weighing 200–250g were procured from Zydus-Cadila Pharmaceuticals, Ahmedabad. The animal house was well ventilated, and rats had 12±1 h day and night cycle with temperature maintained at 25±3 °C and 35–55% relative humidity. Rats were fed with rat pellet feed supplied by Nav-Maharashtra Oil Mills, Maharashtra, India and water *ad libitum*. All the animal experiments were carried out as per the guidelines of Institutional Animal Ethical Committee of Pharmacy Department, The Maharaja Sayaji Rao University of Baroda, Gujarat, India (IAEC Reg. No. 404/01/a/CPCSEA).

2.5 Acute oral toxicity study of fractions

Toxicity study was conducted as per internationally accepted protocol drawn under OECD guidelines 423 in Albino rats of Wistar strain. Group of rats treated with vehicle alone served as a control. The study groups were administered with fractional dose level of 5, 50, 300 and 2000 mg/kg BW, p.o. as a fine suspension in 2% gum acacia.

2.6 Antidiabetic study of fractions

Experimental type 2 diabetes was induced as per Masiello et al.[16] with streptozotocin-nicotinamide (STZ-NAD). The blood glucose levels were determined at 72 h and the rats with fasting blood glucose concentration of more than 180 mg/dL were considered diabetic and selected for the antidiabetic study. The selected animals were divided into 9 groups (n = 6). Group I: normal control rats were administered with 0.5 mL/100 g BW of saline daily; Group II: diabetic control rats were administered with 0.5 mL/100 g BW of saline daily; Group III: diabetic rats were administered with 11.3 mg/kg BW of metformin; Group IV: diabetic rats were administered with USPEF (100 mg/kg BW); Group V: diabetic rats were administered with USPEF (200 mg/kg BW); Group VI: diabetic rats were administered with RFME (100 mg/kg BW); Group VII: diabetic rats were administered with RFME (200 mg/kg BW); Group VIII: diabetic rats were administered with CPF (100 mg/kg BW); Group IX: diabetic rats were administered with CPF (200 mg/kg BW). All the test samples were administered orally once a day for 30 days. The effects of all the test samples were determined by measuring plasma glucose[17], plasma insulin levels[18] and changes in body weight. On day 30 the animals were sacrificed, livers were isolated for the estimation of hexokinase[19], glucose-6-phosphatase[20], glycogen[21], and pancreas was removed for histopathological studies. Liver and pancreas tissues were washed with normal saline and stored in 10% formalin. The pancreatic tissues were processed for paraffin embedding and sections were stained with haematoxylin-eosin reagent. The histological results were recorded as microphotographs and examined for intracellular changes.

2.7 Bioactivity guided isolation by column chromatography

USPEF (2.5 g) was subjected to column chromatography on silica gel 60-120 mesh (100 g) and eluted with gradient mixture of petroleum ether: ethyl acetate (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100). 100 fractions of 25 mL each were collected. The collected fractions were either kept separate or pooled together on the basis of their TLC profile. The fractions with single spot in TLC were further purified by preparative TLC. The six isolated compounds (CPI–VI) were dried and stored in desiccator.

2.8 GC-MS analysis of unsaponified matter of petroleum ether fraction of methanol extract (USPEF)

GC-MS analysis was performed on a GC Clarus 500 (PerkinElmer, USA) instrument equipped with elite-1 column (100% dimethylpolysiloxane), 30 m x 0.25 mm ID x 1 µm df. The column was maintained at 50 °C for 2 min after injection and programmed upto 250 °C at a rate of 5 °C/min and further held for 9 minutes. The temperature of the injection port and interface was set at 280 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min. Sample (2 µL) was injected in the 10:1 split mode. The mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70 eV with a scan mass range of 45-450 atomic mass units. The analytes were identified using the NIST Mass Spectral Database.

2.9 Acute oral toxicity study of isolated compounds

Toxicity study was conducted as per internationally accepted protocol drawn under OECD guidelines 423 in Albino rats of Wistar strain. Isolated compounds were administered at a dose level of 5, 50 and 300 mg/kg BW, p.o. as a fine suspension in 2% gum acacia solution.

2.10 Antidiabetic study of isolated compounds

The isolated compounds CPII and CPVI were studied as described above in section 2.6. The grouping of rats has been as follows: Group I: normal control rats were administered with 0.5 mL/100 g BW of saline daily; Group II: diabetic control rats were administered with 0.5 mL/100 g BW of saline daily; Group III: diabetic rats were administered with 11.3 mg/kg BW of metformin; Group IV: diabetic rats were administered with CPII (15 mg/kg BW); Group V: diabetic rats were administered with CPII (30 mg/kg BW); Group VI: diabetic rats were administered with CPVI (15 mg/kg BW); Group VII: diabetic rats were administered with CPVI (30 mg/kg BW). All the test samples were administered orally once a day for 30 days.

2.11 PTP1B inhibition study

PTP1B inhibitory activity of the samples was tested by using Calbiochem® PTP1B colorimetric assay kit (User Protocol; 2008, Catalogue No: 539736, USA). The absorbance was measured at 620 nm on microplate ELISA reader (BioRad-680XR). The results of the test samples were expressed as amount of phosphate released in nM.

2.12 Antiplatelet aggregation study

Antiplatelet aggregation activity was carried out using heparin-treated whole blood obtained from healthy anaesthetized rats by electrical impedance method using Chrono-Log Model 592VS dual channel whole blood aggregometer (Chrono-Log Corporation, Haverton, PA, USA) as reported[22]. Each reading was taken in triplicate with different concentrations of samples, taking control and the respective concentration of aspirin for comparative reading each time. The 50% inhibition of platelet aggregation was determined for each test sample comparing with control and IC_{50} values were calculated accordingly in µM for isolated compounds or µg/mL for fractions.

2.13 Statistical analysis

The quantitative measurements in all the experiments were made on 6 rats in each group and the values are expressed as mean ± standard deviation. Graphpad Instat Version 4 software was used. Data were subjected to the analysis of variance (one-way ANOVA) to determine the significance of changes followed by Dunnett's test for multiple comparisons.

3 RESULTS

The percentage yield of methanol extract, petroleum ether fraction of methanol extract, USPEF, RFME and CPF were found to be 12.44, 2.03, 0.79, 10.35 and 17.51% w/w with respect to 100 g of air-dried leaves. The elution of USPEF with petroleum ether: ethyl acetate (100:00), (90:10), (80:20), (70:30), (60:40) and (50:50) yielded CPI, CPII, CPIII, CPIV, CPV and CPVI respectively (Figure 1).

The structures of isolated compounds were confirmed by spectral analysis such as IR, ^{1}H NMR, ^{13}C NMR, mass spectra and CHNOS analysis, and were found to be in accordance with the structures reported in the literature. CPI, CPIII, CPIV and CPV were characterized as 1-hexadecanol, β-carotene, β-sitosterol[23] and lupeol[23] respectively. CPII[24] and CPVI were characterised as clerosteryl palmitate and clerosterol respectively (Figure 2 and 3).

The acute toxicity study was performed for establishing the therapeutic index and for the primary screening. The fractions showed no signs of toxicity up to the dose of 2000 mg/kg. The isolated compounds clerosteryl palmitate (CPII) and clerosterol (CPVI) also showed no signs of toxicity up to the dose of 300 mg/kg.

USPEF 100 mg/kg, USPEF 200 mg/kg, CPF 200 mg/kg, CPII 30 mg/kg, CPVI 30 mg/kg treated groups showed significant change ($p < 0.01$) in restoring the glucose and insulin levels (Table 1), in comparison to the control group.

Interestingly, USPEF 200 mg/kg and CPVI 30 mg/kg treated groups showed better restoration of both glucose and insulin levels than the other treated groups. Metformin, USPEF 100 mg/kg, 200 mg/kg and CPVI 30 mg/kg treated groups showed a significant ($p < 0.01$) increase while CPII 30 mg/kg and CPVI 15 mg/kg treated groups showed less significant ($p < 0.05$) increase in hexokinase level (Table 2). Metformin, USPEF 200 mg/kg, CPII 30 mg/kg and CPVI 30 mg/kg treated groups showed significant ($p < 0.01$) decreased activity of glucose-6-phosphatase (Table 2).

The hepatic glycogen content of diabetic control is reduced significantly as compared to all other treated groups. Metformin, USPEF 100 mg/kg, USPEF 200 mg/kg, CPVI 30 mg/kg and CPII 30 mg/kg treated groups showed significant ($p < 0.01$) changes among which USPEF 200 mg/kg and CPVI 30 mg/kg treated groups restored the depleted glycogen level better (Table 2). Metformin and USPEF 200 mg/kg treated groups

showed significant change ($p < 0.01$) in restoring the body weight (Table 3).

Diabetic control group (Figure 4b) showed prominent disruption of the islet cellular architecture with large clear vacuoles compared to normal control group (Figure 4a). The metformin treated group (Figure 4c) showed significant higher number of cells per islet and the cellular architecture was preserved. It also showed no vacuoles and degranulation. RFME (100 and 200 mg/kg) (Figure 4f and 4g), CPF (100 and 200 mg/kg) (Figure 4h and 4i), and CPII (15 mg/kg) (Figure 4j) treated groups showed similar observation as that of diabetic control with no significant change. CPII (30 mg/kg) (Figure 4k) and CPVI (15 and 30 mg/kg) treated groups (Figure 4l and 4m) showed shrunken islets with disrupted cellular architecture and significant reduction in total number of cells per islet, but showed comparatively less vacuoles. USPEF (100 and 200 mg/kg) treated groups (Figure 4d and 4e) showed relatively intact, larger size islets, significant number of cells per islet and reduced vacuoles due to its rejuvenation of β-cells leading to increase in insulin production and secretion.

The GC-MS analysis of bioactive USPEF shows the retention time and peak area of various compounds detected as shown in Figure 5. Based on their retention time and molecular weight, compounds were matched from library database (NIST Mass Spectral Database). 1,2-Benzenedicarboxylic acid diisooctyl ester ($C_{24}H_{38}O_4$) was found to be the compound with highest peak area of 74.80%. Other compounds identified with significant peak area were phytol ($C_{20}H_{40}O$, 8.40%), vitamin E ($C_{29}H_{50}O_2$, 6.44%) and 8,11,14-eicosatrienoic acid ($C_{20}H_{34}O$, 1.30%) (Table 4).

Suramin is a reversible and competitive inhibitor of PTP1B, with a K_i of 5.5 µM. Clerosterol (CPVI) showed the highest potency with IC_{50} of 23.28±1.6 µM and clerosteryl palmitate (CPII) showed an IC_{50} of 32.23±0.8 µM among the isolated compounds. USPEF showed the lowest IC_{50} of 23.50±1.1 µg/mL among the fractions studied (Table 5).

Clerosteryl palmitate (CPII) exhibited the highest potency (IC_{50} = 27.49±3.3 µM) in ADP induced platelet aggregation among the isolated compounds and USPEF (IC_{50} = 11.12±0.8 µg/mL) among the fractions studied (Table 6).

Table 1 Changes in plasma glucose and insulin levels in normal control, standard, diabetic control and treated groups

Groups	Plasma glucose (mg/dl)	Plasma insulin (µU/ml)
Normal control	87.04 ± 4.86 **	16.59 ± 2.02 **
Diabetic control	275.34 ± 28.86	4.86 ± 1.05
Metformin (11.3 mg/kg)	145.26 ± 24.22 **	14.56 ± 1.54 **
USPEF (100 mg/kg)	238.04 ± 14.14 **	9.35 ± 0.56 **
USPEF (200 mg/kg)	188.55 ± 10.32 **	11.24 ± 0.98 **
RFME (100 mg/kg)	253.82 ± 21.29 ^{ns}	6.29 ± 1.95 ^{ns}
RFME (200 mg/kg)	255.19 ± 19.02 ^{ns}	6.5 ± 1.37 ^{ns}
CPF (100 mg/kg)	249.14 ± 11.83 ^{ns}	7.01 ± 0.67 ^{ns}
CPF (200 mg/kg)	240.94 ± 8.39 **	7.81 ± 1.07 **
CPII (15 mg/kg)	255.81 ± 10.13 ^{ns}	7.54 ± 2.04 **
CPII (30 mg/kg)	235.92 ± 10.22 **	9.76 ± 1.11 **
CPVI (15 mg/kg)	220.38 ± 19.66 **	7.48 ± 1.23 *
CPVI (30 mg/kg)	207.10 ± 11.78 **	10.13 ± 1.38 **

Values are expressed as Mean ± SD; n = 6; * $p < 0.05$; ** $p < 0.01$; ^{ns} not significant

Table 2 Changes in hepatic hexokinase, glucose-6-phosphatase and liver glycogen levels in normal control, standard, diabetic control and treated groups

Groups	Hexokinase (U/g/Tissue)	Glucose-6-phosphatase (U/g/min/Tissue)	Liver glycogen (µg of glucose/mg of wet tissue)
Normal control	10.51 ± 1.97 **	14.82 ± 2.57 **	53.78 ± 2.18 **
Diabetic control	2.24 ± 1.69	35.38 ± 6.54	25.13 ± 3.21
Metformin (11. 3mg/kg)	8.54 ± 1.1 **	19.35 ± 5.67 **	52.24 ± 1.28 **
USPEF (100 mg/kg)	5.96 ± 1.71 **	26.38 ± 3.98 *	30.69 ± 4.58 **
USPEF (200 mg/kg)	7.05 ± 0.54 **	23.14 ± 4.31 **	45.21 ± 1.29 **
RFME (100 mg/kg)	3.54 ± 0.85 ^{ns}	29.94 ± 5.41 ^{ns}	26.98 ± 2.67 ^{ns}
RFME (200 mg/kg)	3.91 ± 0.67 ^{ns}	28.10 ± 2.01 ^{ns}	28.34 ± 1.68 ^{ns}
CPF (100 mg/kg)	3.68 ± 1.27 ^{ns}	31.02 ± 8.14 ^{ns}	28.96 ± 2.31 ^{ns}
CPF (200 mg/kg)	4.18 ± 1.67 ^{ns}	29.68 ± 2.37 ^{ns}	30.34 ± 1.39 *
CPII (15 mg/kg)	4.09 ± 2.01 ^{ns}	29.27 ± 6.98 ^{ns}	29.67 ± 3.01 *
CPII (30 mg/kg)	4.59 ± 1.91 *	24.68 ± 6.98 **	31.29 ± 1.29 **
CPVI (15 mg/kg)	4.68 ± 1.26 *	29.64 ± 2.57 ^{ns}	29.68 ± 2.67 *
CPVI (30 mg/kg)	5.96 ± 1.35 **	24.08 ± 7.12 **	39.16 ± 3.67 **

Values are expressed as Mean ± SD; n = 6; * $p < 0.05$; ** $p < 0.01$; ^{ns} not significant.

Table 3 Changes in body weight in normal control, standard, diabetic control and treated groups.

Groups	Body weight ^{a} (g)
Normal control	226.13 ± 4.57 **
Diabetic control	165.24 ± 9.56
Metformin (11.3 mg/kg)	220.35 ± 4.87 **
USPEF (100 mg/kg)	171.64 ± 4.57 ^{ns}
USPEF (200 mg/kg)	190.65 ± 7.13 **
RFME (100 mg/kg)	171.37 ± 5.67 ^{ns}
RFME (200 mg/kg)	170.35 ± 6.98 ^{ns}
CPF (100 mg/kg)	168.25 ± 8.51 ^{ns}
CPF (200 mg/kg)	169.12 ± 4.87 ^{ns}
CPII (15 mg/kg)	164.24 ± 5.49 ^{ns}
CPII (30 mg/kg)	170.24 ± 5.97 ^{ns}
CPVI (15 mg/kg)	168.38 ± 9.37 ^{ns}
CPVI (30 mg/kg)	170.58 ± 7.49 ^{ns}

Values are expressed as Mean ± SD; n = 6; ** $p < 0.01$; ^{ns} not significant; ^{a} : rat wt. range = 225 ± 10 g.

Table 4 Compounds identified by GC-MS using NIST mass library in USPEF of *C. phlomidis* leaves

No.	R. T. (min)	Name of the Compounds identified	Mol. Formula	Mol. Wt.	Area (%)
1	3.10	1,3,5,7-Cyclooctatetraene	C_8H_8	104	0.15
2	25.33	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	0.76
3	28.19	Phytol	$C_{20}H_{40}O$	296	8.40
4	28.61	8,11,14-Eicosatrienoic acid	$C_{20}H_{34}O$	306	1.30
5	34.71	Vitamin E	$C_{29}H_{50}O_2$	430	6.44
6	35.17	1,2-Benzenedicarboxylic acid diisooctyl ester	$C_{24}H_{38}O_4$	390	74.80

Table 5 PTP1B inhibitory effect of fractions and the isolated compounds.

Fractions/isolated compounds	IC_{50} (µM)
Suramin [a]	5.37±0.6
USPEF	23.50±1.1 [b]
RFME	26.33±1.7 [b]
CPF	25.13±1.4 [b]
CPII	32.23±0.8
CPVI	23.28±1.6

Values are expressed as Mean±SD; [a] Positive control; [b] µg/ml.

Table 6 Effects of fractions/isolated compounds on ADP induced platelet aggregation.

Fractions/isolated compounds	IC_{50} (µM)
Aspirin [a]	10.14±0.7
USPEF	11.12±0.8 [b]
RFME	Aggregation Inducer
CPF	24.72±3.4 [b]
CPII	27.49±3.3
CPVI	36.75±2.6

Values are expressed as Mean±SD; [a] Positive control; [b] µg/ml.

Figure 1 Bioactivity guided fractionation of *Clerodendrum phlomidis* leaves

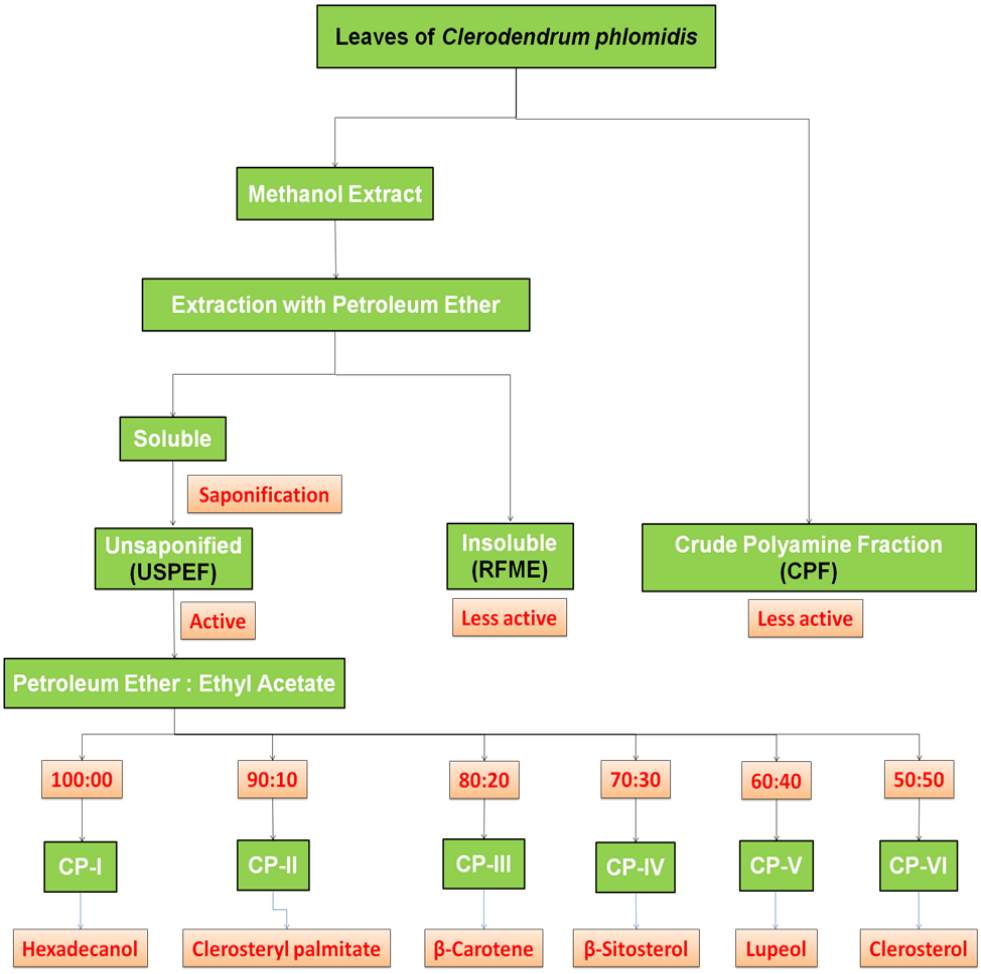


Figure 2 Chemical structure of CP II

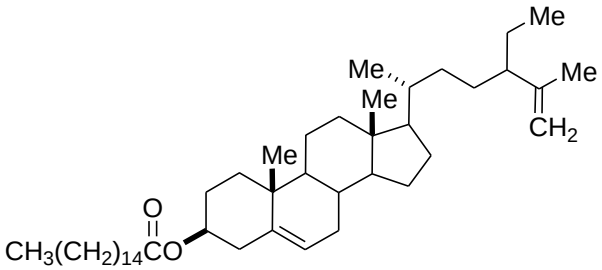


Figure 3 Chemical structure of CP VI

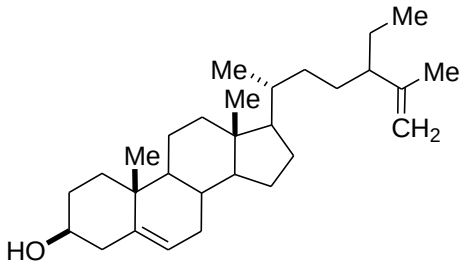
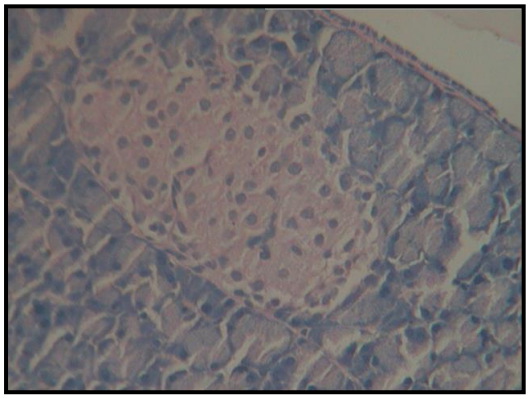
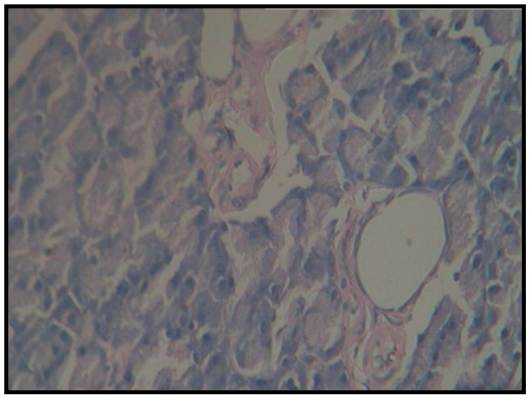


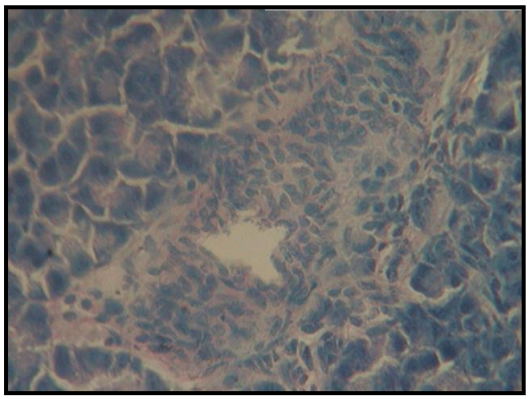
Figure 4 Changes in the endocrine (islets of Langerhans) and exocrine pancreas (acini) of normal control, standard, diabetic control and treated groups



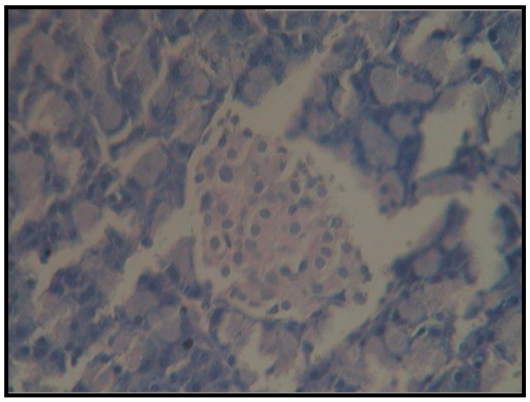
(a) Normal control group



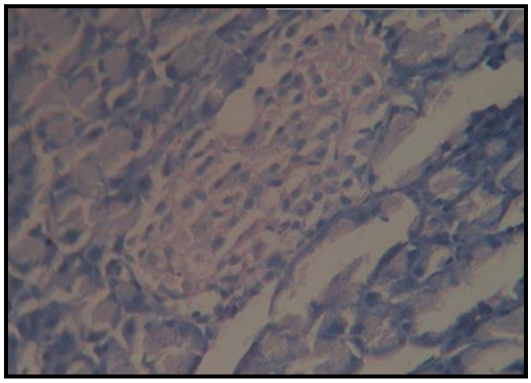
(b) Diabetic control group



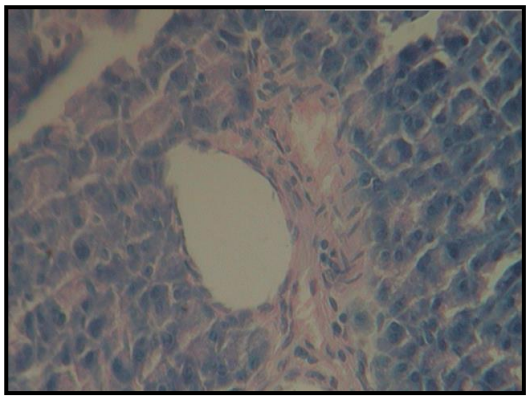
(c) Metformin (11.3 mg/kg) treated group



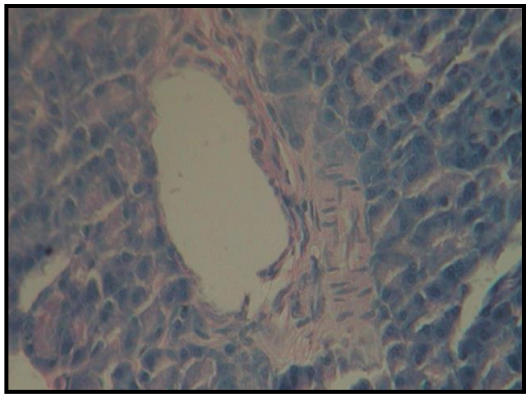
(d) USPEF (100 mg/kg) treated group



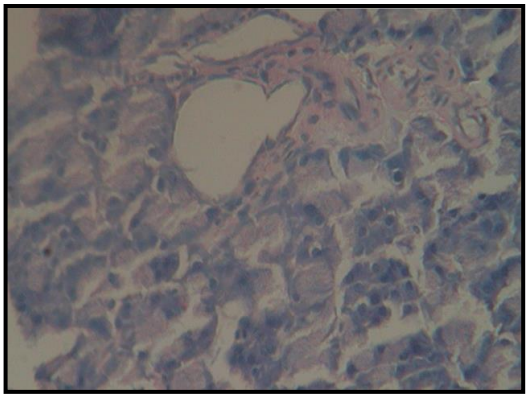
(e) USPEF (200 mg/kg) treated group



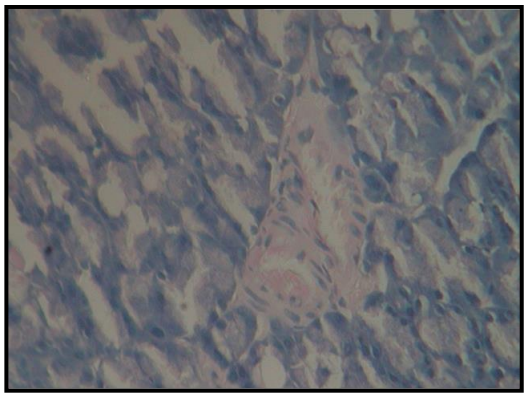
(f) RFME (100 mg/kg) treated group



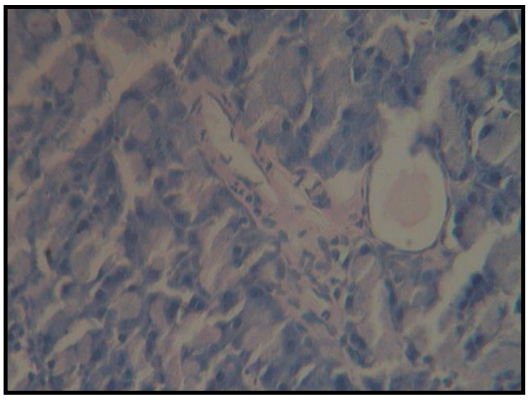
(g) RFME (200 mg/kg) treated group



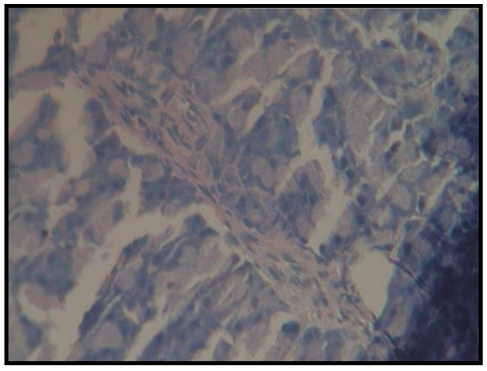
(h) CPF (100 mg/kg) treated group



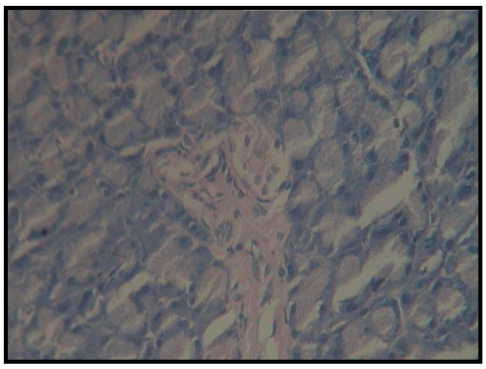
(i) CPF (200 mg/kg) treated group



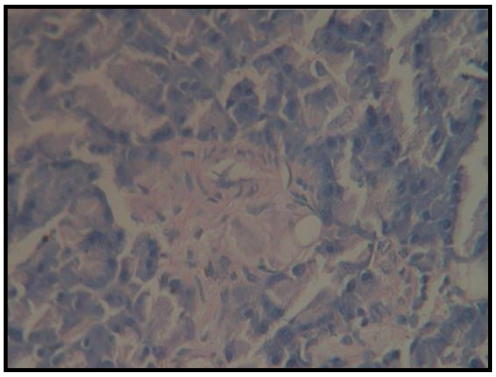
(j) CPII (15 mg/kg) treated group



(k) CPII (30 mg/kg) treated group

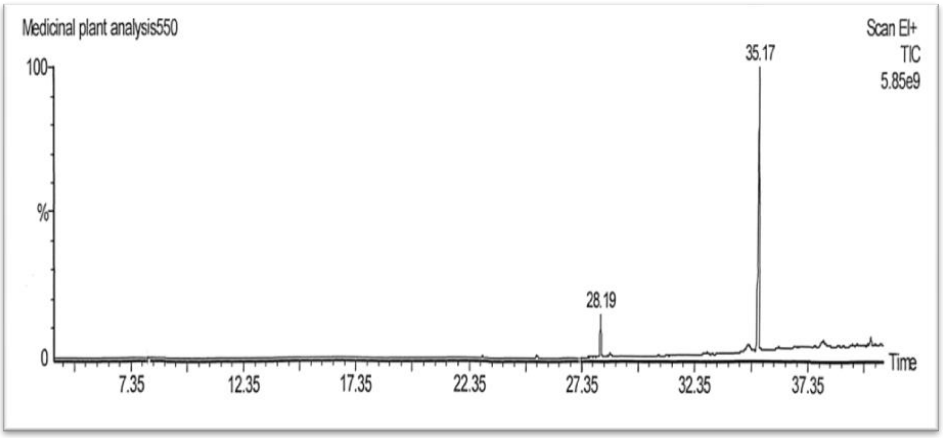


(l) CPVI (15 mg/kg) treated group



(m) CPVI (30 mg/kg) treated group

Figure 5 Gas chromatogram of USPEF of C. *phlomidis* leaves



4 DISCUSSION

Polyamines have been identified as a new target for islet β-cell protection and platelet aggregation inhibition[25-28]. Preliminary qualitative phytochemical study indicated large quantities of polyamines in leaves of *C. phlomidis*. Hence crude polyamine fraction (CPF) was prepared and evaluated.

The antidiabetic study of fractions showed USPEF as the most active fraction obtained, hence this fraction was chosen for phytochemical investigation. Clerosteryl palmitate (CPII) and clerosterol (CPVI) are reported for the first time from the leaves of *C. phlomidis.* Clerosterol (CPVI) has been previously reported from the roots[29] of *C. phlomidis*. The ubiquitous occurrence of 1-hexadecanol (CPI), β-carotene (CPIII), β-sitosterol (CPIV) and lupeol (CP V) in general and their glucosides in all vegetables make it highly unlikely that they have any drug related properties. Moreover, reports on their medicinal properties are based on *in vitro* or unrealistically high *in vivo* doses which make a therapeutic application of these compounds highly unlikely[30]. Hence 1-hexadecanol (CPI), β-carotene (CPIII), β-sitosterol (CPIV) and lupeol (CPV) were ignored for further study.

The diabetic control group showed a marked increase in plasma glucose and a significant reduction in insulin level while the metformin treated group restored the elevated glucose level to near normal level and also increased the reduced insulin level. The significant decrease in the glucose levels and increase in the insulin levels of diabetic rats treated with USPEF 200 mg/kg and CPVI 30 mg/kg shows the stimulation of insulin secretion from the remnant β-cells. Some studies[31] have reported that antidiabetic plants may affect circulating insulin levels, indicating that USPEF 200 mg/kg and CPVI 30 mg/kg also might have induced the release of insulin from the bound form.

Hexokinase, a key glycolytic enzyme responsible for the first phosphorylation step of glucose metabolism, is reduced significantly in the diabetic rats[32,33]. The activation of glycolysis and increased utilization of glucose for energy production are the reasons for the significant increase of hexokinase in USPEF 100 mg/kg, 200 mg/kg and CPVI 30 mg/kg treated groups. But enhancement of hexokinase activity may also be due to glycogen synthesis activation or activation of mRNA coding for hexokinase in diabetic rats[32].

The increased activity of glucose-6-phosphatase in the liver of diabetic rats is due to the increased glucose production[35]. The decreased levels of glucose-6-phosphatase observed in USPEF 200 mg/kg, CPII 30 mg/kg and CPVI 30 mg/kg treated diabetic animals are primarily due to gluconeogenic enzyme activity regulation through the regulation by cAMP, metabolic activation of glycolysis and gluconeogenesis.

The reduced glycogen store in diabetic rats has been attributed to reduced activity of glycogen synthase[36], increased activity of glycogen phosphorylase[37] and insulin deficiency[38]. Many studies have also demonstrated that impaired insulin sensitivity or hyperglycemic or hyperinsulinemia affects glucose storage in liver and muscle[39,40]. USPEF 200 mg/kg and CPVI 30 mg/kg treated groups prevented the alteration in glycogen content but could not normalise it. This prevention of depletion of glycogen in liver is due to decreased activity of glycogen phosphorylase and increased activity of glycogen synthase. Accumulation of glycogen in liver of USPEF 200 mg/kg and CPVI 30

mg/kg treated animals is similar to insulin therapy[41]. However, USPEF 200 mg/kg and CPVI 30 mg/kg can also act by stimulating insulin release from β-cells[42] and mimicking insulin effect[43]. Therefore, USPEF 200 mg/kg and CPVI 30 mg/kg appear to exert both pancreatic and extra pancreatic effects.

STZ-NAD induced diabetes is associated with a characteristic loss of body weight which is due to increased muscle wasting in diabetic state[44]. The reversal of weight loss in the USPEF 200 mg/kg treated diabetic rats indicates the reversal of gluconeogenesis and glycogenolysis. The resumption of moderate body weight also indicates improvement in sugar and lipid metabolism.

Significant reduction in total number of cells per pancreatic islet with marked degranulation in diabetic control was observed. This provides clear evidence that STZ-NAD destroyed the pancreatic β-cells. Rejuvenated histopathological profile for USPEF (100 and 200 mg/kg) treated groups is due to its regeneration of β-cells leading to increase in insulin production and secretion.

The overall results showed that RFME is completely inactive. CPF reduced glucose levels, increased glycogen levels, but showed no significant change in insulin, hexokinase and glucose-6-phosphatase levels. Histopathological study of CPF showed no effect of stimulating insulin secretion from the remnant β-cells or regenerated β-cells. The plasma glucose lowering effect in the absence of a significant change in plasma insulin concentration indicates that the CPF additionally involves an insulin-independent-mechanism. CPF exhibits this insulin-independent-mechanism by an extra-pancreatic action *i.e.*, by stimulating glucose utilisation in peripheral tissues and an increase in glycolytic and/or glycogenic enzymes activity.

CPII and CPVI have shown moderate effects on glucose, insulin, hexokinase, glucose-6-phosphatase and glycogen but caused no increase in body weight. Their histolopathological studies show very modest effect or nil effect. Absence of weight gain indicates the non-reversal of gluconeogenesis and glycogenolysis. The observed hypoglycemic effect is either due to insulinomimetic effect or by increasing peripheral utilization of glucose or increased glycogen synthase or increased glycolysis. CPII being the palmityl ester of CPVI explains the parallel pattern of activity but CPVI has shown comparatively better antidiabetic activity.

USPEF has shown nearly comparable effect to that of metformin on all the parameters studied. Additionally, β-carotene, β-sitosterol and lupeol (CPIII, CPIV and CPV) isolated from USPEF, have been reported individually for antioxidant activity, insulin releasing effect, antihyperglycemic effect, hypolipidemic effect and β-cell regeneration. The current study has shown moderate antidiabetic activity of CPII and CPVI. Synergistic effect is the only possible explanation for the better antidiabetic activity of USPEF than all the studied samples.

The bioactive USPEF was further subjected to GC-MS analysis. The beneficial effect of vitamin E in diabetic patients and its complications has been reported[45]. The conversion of phytol to phytanic acid is regulated *via* peroxisome proliferator-activated receptor-α (PPARα) and phytanic acid being a natural PPAR agonist, regulates glucose metabolism in rat primary hepatocytes[46]. Based on these observations, hypoglycemic effect exhibited by USPEF may also be due to the presence of vitamin E and phytol.

PTP1B inhibitors through insulin signaling could potentially ameliorate insulin resistance; normalize plasma glucose and insulin without inducing hypoglycemia. The potential PTP1B inhibition of CPVI suggests that the presence of fatty acid chain has hindered the PTP1B inhibition of CPII. The constituents of USPEF, β-sitosterol (CPIV)[47] and β-carotene (CPIII)[48] have been reported for less/no PTP1B inhibition.

Adenosine diphosphate (ADP) causes a full range of activation events including intraplatelet Ca^{2+} elevation, TxA_2 synthesis, protein phosphorylation, granule secretion, activation of glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) and aggregation. All these events are mediated by interaction with two classes of purinergic G protein coupled receptors (GPCR), P2Y purinoceptor 1 ($P2Y_1$) and P2Y purinoceptor 12 ($P2Y_{12}$)[49]. Stimulation of $P2Y_1$ activates phopholipase C, whereas stimulation of $P2Y_{12}$ down regulates adenylate cyclase activity, leading to lower levels on cAMP, an inhibitor of platelet activation. At cellular level, stimulation of $P2Y_1$ generates an initial transient aggregation response, whereas agonism of $P2Y_{12}$ generates sustained aggregation. Cyclooxygenase-1 (COX-1) in the platelet normally converts arachidonic acid (AA) to prostaglandin H_2 (PGH_2), a precursor of the potent platelet activator TxA_2. Aspirin irreversibly inactivates COX-1 by acetylating the hydroxy group of Ser-529 near the

active site, thereby blocking the binding of its substrate (AA). Adrenaline, a known platelet sensitizer and antagonist of insulin, abolishes the effect of insulin on $[Ca^{2+}]_i$ tyrosine phosphorylation of $Gi\alpha_2$ and causes aggregation. Although ADP initiates aggregation, adrenaline also plays a prominent role in the process of extension of the platelet plug[50]. Adrenaline has been reported in *C. phlomidis* leaves[51]; hence RFME increases overall platelet aggregation. Significant inhibition of ADP induced platelet aggregation by USPEF and CPII are due to activation of $P2Y_1/P2Y_{12}$. The potential antiplatelet aggregation of USPEF is also due to its constituents, β-Sitosterol (CPIV)[52] and vitamin E[45] which have been previously reported for anti-platelet and anti-thromboxane activities respectively.

CPII and CPVI showed moderate antidiabetic and antiplatelet effects. The results indicated that USPEF has potent hypoglycemic (*in vitro* and *in vivo*) and antiplatelet effect than the isolated compounds. It was concluded that USPEF exhibits synergistic antidiabetic and antiplatelet activities.

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