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Analyses and profiling of extract and fractions of neglected weed *Mimosa pudica* Linn. traditionally used in Southeast Asia to treat diabetes

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ABSTRACT

Mimosa pudica Linn. var. *hispida* Bren. (Family: Fabaceae) a neglected weed has been studied for its antidiabetic potential to propose alternative medicinal source against the global threat of diabetes mellitus. This study aimed to investigate *in vitro* inhibitory activity against diabetic enzymes (i.e. α-amylase & α-glucosidase) and three anti-oxidant assays were conducted to evaluate anti-diabetic potential of *M. pudica's* methanol extract ($MeOH_i$) and its sub-fractions ($Hexane_f$, $EtOAc_f$, $Acetone_f$ and $MeOH_f$). In depth chemical profiling using GC Q-TOF MS was also performed for the first time for this weed, to assess the probable compounds present in the extract and sub fractions that could be linked to anti-diabetic activity. Results showed the lowest (7.18 ± 0.0005) and highest (158.4 ± 0.0004) IC_{50} for DPPH assay by $MeOH_i$ and $MeOH_f$, respectively. $Acetone_f$ and $MeOH_i$ showed the highest TPC (60.07 ± 1.066) and TFC (16.97 ± 1.472), respectively. Three and two fold higher inhibitory activity than the standard acarbose at 1 mg/ mL was manifested by $MeOH_i$ (95.65 ± 0.911) & $EtOAc_f$ (51.87 ± 3.106), respectively. $Hexane_f$ did not show inhibitory activity against both the enzymes. α-glucosidase results for the extract and sub fractions were found to be significant ($p < 0.05$). GC Q-TOF MS analysis identified organic acids, quinolones, quinone, phenolic compounds and dodecaborane as major constituents. Presence of highly radical scavenging dodecaborane is being reported for the first time in *M. pudica*. High TPC and TFC values could be attributed to exert enzyme inhibitory action by *M. pudica* that can help in the regulation of glucose absorption and consequently glucose homeostasis. Results show that *M. pudica* can be proposed as an excellent alternative for future anti-diabetic implications.

1. Introduction

Diabetes mellitus (DM) is a non-communicable disease often genetic in nature but can be developed due to life style mishaps. DM often comes with a plethora of other related complications like heart disease, stroke, atherosclerosis (Macroangiopathy), neuropathy leading to diabetic wound, nephropathy leading to kidney failure, retinopathy leading to glaucoma or blindness (Microangiopathy) (ADA, 2004; WHO, 2013).

More than 80% of diabetic patients are residents of low or middle income countries where the primary mode of treatment is still alternative medicinal modes like traditional herbal, natural medicine or homeopathy (WHO Traditional Medicinal Strategies, 2002-2005; Mathers and Loncar, 2006). 65%- 80% of world's population directly or indirectly depend on alternative medicinal forms. Such massive use clearly proves the efficiency and benefits of herbal medicines over or in contrast to modern drugs system. However, wide use of medicinal plants throughout the world further adds stress on the flora of the world's vegetative populations and the ramifications can be threatening if not taken into account for alternative forms of medicinal plants to reduce stress on the flora of the world's medicinal plants. In lieu to that weeds could be proposed as alternative medicine and could be a bounty of novel drug leads as well (Stepp and Moerman, 2001; WHO Traditional Medicinal Strategies, 2002-2005; Stepp, 2004).

Traditional medicines have been used to treat diabetes mellitus since time immemorial (Grover et al., 2002). Natural medicines have been reported to show anti-diabetic activity through different mechanisms. Currently available anti-diabetic drugs exert their activity through various mechanisms. One such is the controlling of post-prandial glucose in blood. Plants have been reported to reduce the absorption of glucose by retarding the digestive enzymes i.e. α-amylase and α-glucosidase from pancreas and gut which in-turn helps preventing the sky-rocketing of postprandial glucose in the blood (Oboh et al., 2012; Djeridane et al., 2013; Kumar et al., 2013; Yao et al., 2013). Inhibition of the aforementioned enzymes has been positively linked with antioxidants specially flavonoids and phenolic compounds (Wang et al., 2012). Natural antioxidants like phenolic compounds and flavonoids have been shown to prevent oxidative stress, thereby, reducing the onset and prognosis of diseases like diabetes mellitus (Djeridane et al., 2013). The natural antioxidants, apart from preventing oxidative stress can also display

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digestive enzymes inhibitory activities (Mai et al., 2007; Wang et al., 2012; Djeridane et al., 2013). These findings prompted us to carry out current study to investigate the antioxidant profiling through detecting the total flavonoid content (TFC), phenolic content (TPC), radical scavenging activity (DPPH), α-amylase and α-glucosidase inhibitory activity of the chosen sample (*M. pudica*).

In view to the quest of proposing alternative source that will be rich in anti-diabetic compounds to fight diabetes mellitus and its related complications, *Mimosa pudica* Linn. var. *hispida* Bren. a common neglected weed with traditional anti-diabetic and other benefits has been studied. It belongs to Fabaceae family and is commonly distributed in roadside, walkway, marsh and hillside areas. Traditionally, it is effectively used to treat diabetes mellitus, as a wound healing agent, antimicrobial, anti-venom, anti-oxidative, anti-cancer, anti-infectious etc. Previously some scientific studies have been reported to confirm its aforementioned traditional claims through *in vivo* and *in vitro* studies (Umamaheswari and Prince, 2007; Sutar et al., 2009; Manosroi et al., 2011; Suganthi et al., 2011; Zhang et al., 2011). However, in the current study, it has been aimed to evaluate its *in vitro* anti-diabetic activity against two digestive enzymes viz. α-amylase and α-glucosidase for the first time. The aim of this study was also to perform phytochemical screening using chemical methods and then establish the anti-diabetic efficacy of *M. pudica* through DPPH, total phenolic content assay (TPC), total flavonoid content assay (TFC) and enzyme inhibitory assays followed by GCMS determination through GC Q-TOF MS analysis. This is the first ever report of α- amylase and α- glucosidase inhibitory activity analysis for this plant as well as GC Q-TOF MS analysis for the extract and fractions.

2. Materials and methods

2.1. Chemicals and reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin Ciocalteu reagent and solvents were bought from Fisher and Merck. The enzymes (α-amylase type iv- B from porcine pancreas and α-glucosidase type 1 from baker's yeast), p-nitrophenyl-α-D-glucopyranoside (ρ-NDG), 3,5-dinitro salicylic acid (DNS), potato starch, sodium phosphate, potassium phosphate monobasic, dipotassium phosphate, NaCl, Na_2CO_3 etc were bought from Sigma-Aldrich. Ultra pure water was used wherever water was needed as the solvent. UV-Vis Microplate Spectrophotometer from TecanNanoQuant, Infinite M200, Austria was used.

2.2. Plant material

Fresh aerial parts of *M. pudica* were collected during flowering season (March till July 2013) from the vicinity of the International Islamic University Malaysia campus (IIUM), Kuantan, Pahang DM, Malaysia. The plant was identified by Dr. Norazian Hassan (Taxonomist), KOP, IIUM, Malaysia. The voucher specimen (NMPC-QU037) has been deposited in the Herbarium, Faculty of Pharmacy, IIUM, Kuantan, Pahang DM, Malaysia for future references.

2.3. Extraction and fractionation

The fresh aerial parts of *M. pudica* (9.5 kg) were dried in a PROTECH laboratory air dryer (FDD-720-Malaysia) at 40 °C for 7 days and pulverized using Fritsch Universal Cutting Mill-PULVERISETTE 19-Germany. It was then stored in a dessicator at 2 °C until further use. The coarsely ground dry powder [3.1 kg (32.63%)] was taken and subjected to 3 cycles (each 24 h soaking) of methanol extraction at room temperature followed by 4 cycles of heat under reflux (2 h each) over boiling water bath. The extract was filtered and concentrated using a rotary evaporator (Buchi Rotary Evaporator, R-210, Switzerland). Final concentrated methanol extract ($MeOH_i$) (mother extract) upon drying was successively extracted to fractionation with hexane, ethyl acetate, acetone and methanol successively to get hexane fraction ($Hexane_f$), ethyl acetate fraction ($EtOAc_f$), acetone fraction ($Acetone_f$) and methanol fraction ($MeOH_f$), respectively based on their increasing polarity (Umar et al., 2010). All extracts were stored at 2 °C in labeled sterile bottles and kept as aliquots until further anti-diabetic evaluation. All chemicals used in this study were of analytical grade and double distilled.

2.4. Phytochemical and GCMS screening

All extracts of *M. pudica* were subjected to phytochemical screening using dried samples for the presence of different classes of organic compounds like alkaloids, flavonoids, terpenoids, coumarine, saponine, anthraquinone etc. The results are shown in Table 1. Gas Chromatography-Mass Spectra with Time of Flight (GCMS-Q-TOF) technology was employed for the chemical profiling of the extracts and sub-fractions prepared. The method has been discussed in subsection 2.5.6 and 2.5.7.

2.5. In vitro analysis

2.5.1. DPPH free radical scavenging activity

This assay was carried out to determine the free radical scavenging potential of a sample with respect to its inhibitory effects against DPPH free radical. The initial mother extract ($MeOH_i$) and subsequent sub-fractions ($Hexane_f$, $EtOAc_f$, $Acetone_f$ and $MeOH_f$) were evaluated for their free radical scavenging activity using DPPH free radical scavenging assay by following the method described by Nickavar et al., 2006 with some modifications. In short, 1 mL of various concentrations of $MeOH_i$ and fractions in methanol (3–100 μg/mL) were prepared and treated with 2 mL of 0.1 mM of DPPH (prepared fresh with methanol) and diluted using 1 mL of ultrapure water. The mixture was kept in an incubator at 30 °C (found to be optimum) for 30 min after which absorbance was taken using a UV spectroscope at 517 nm. Methanol was employed as blank and DPPH, methanol and water (2:1:1) were employed as controls. Quercetin was used as standard and IC_{50} values in μg/mL were determined for all the samples and standard deviation was calculated. Percentage DPPH scavenging activity was calculated using the following equation:

$$\%\ \text{Scavenging activity} = (\text{Control} - \text{Absorbance})/\ \text{Control x } 100 \quad (1)$$

2.5.2. Total phenolic content (TPC) assay

This assay was followed to determine the total amount of phenolic compounds present in the sample with respect to a standard phenolic compound (Gallic acid). TPC was determined using Folin Ciocalteu (FC) method by following the procedure described by Singleton and Rossi (1965). To evaluate the TPC of the initial extract ($MeOH_i$) and subsequent sub-fractions ($Hexane_f$, $EtOAc_f$, $Acetone_f$ and $MeOH_f$), each of the samples (0.5 mL) was mixed with 2.5 mL of FC reagent (10 times dilution with deionized (DI) water) in amber glass vials and kept aside for 6 min. Subsequently, 2 mL of 7.5% Na_2CO_3 was added and the media was vortexed and then kept for incubation at 30 °C for 30 min. After incubation, the supernatants were collected and the absorbance was taken using UV-Vis spectrometer at 760 nm. Experiments were performed in triplicate. Gallic acid was used as the standard and the TPC was calculated using the following equation

$$\text{TPC (mg/ g)} = \text{GAE x V x } \left(\text{D x}10^{-6}\text{x } 100\right)/\text{Sw} \quad (2)$$

GAE-gallic acid equivalent (mg); V = Vol. of sample (mL); D = dilution factor; Sw = sample weight in grams.

Table 1
Phytochemical screening for chemical class identification of *M. pudica*.

Bioactive	Test/procedure	Observation	Inference
Alkaloids	Dragendorff's reagent, Mayer's reagent	Orange ppt.	+ve
		Turbity	+ve
Saponine	Frothing test,	Very little frothing, little turbidity	+ve (scanty)
	Emulsion		+ve (scanty)
Terpenoids	Salkowski test	Reddish brown ring at the junction	+ve
	Chloroform + H_2SO_4 (absolute)		
Flavonoids	Shinoda test	Solution turns:	+ve
	Flavones: Mg filling	Reddish brown	+ve
	Flavonols: Zn pellet	Amber	+ve
	Flavonoids: NH_3 + H_2SO_4	Yellow	
Carotenoids	Chloroform + H_2SO_4, (85%)	No blue colour	-ve
Free Anthraquinone	Chloroform + NH_3 (10%)	No change from dirty green	-ve
Combined Anthraquinone	10% HCl + Chloroform	No change from colourless	-ve
Coumarine	Filter paper soaked in 1 N NaOH and placed on the sample, heated	Yellow ring	+ve

2.5.3. Total flavonoid content (TFC) assay

This assay was followed to determine the total amount of flavonoids present in a particular sample with respect to a standard flavonoid (Quercetin). TFC assay was performed using $AlCl_3$ colorimetric method by following the method described by Zhishen et al. (1999). In amber glass tubes, 500 μL of extract/fractions were mixed with 2 mL DI water and 15 μL of 5% $NaNO_3$ and incubated at room temperature for 6 min. Subsequently, 150 μL of 10% $AlCl_3$, 2 mL of 2 M NaOH and 200 μL of water were added. The reaction media was vortexed and incubated at 30 °C for 30 min. After incubation, absorbance was measured at 415 nm. Quercetin was used as standard and appropriate blanks were used. Experiments were done in triplicate. TFC was calculated using the following equation:

$$\text{TFC (mg/g)} = \text{QE x V x } \left(\text{D x } 10^{-6}\text{x } 100\right)/\text{Sw} \quad (3)$$

GAE- gallic acid equivalent (mg); V = Vol. of sample; D = dilution factor; Sw = sample weight in grams.

2.5.4. α-amylase inhibitory assay

Enzyme inhibitory assay for α-amylase was performed by following the standard protocols from Worthington (1993) with some modifications. In 96-well plate, 25 μL of each sample (1 mg/mL) was added to 25 μL of (0.5 mg/mL) α-amylase enzyme (Sigma-Aldrich) in 20 mM sodium phosphate buffer (pH 6.9, with 6.7 mM NaCl). The mixture was incubated at 25 °C for 10 minutes followed by addition of 25 μL of 0.5% starch in phosphate buffer. The mixture was further incubated at 25 °C for 10 min. Subsequently, reaction was stopped using 50 μL of DNS solution and incubated over water bath at 100 °C for 5 min. Readings were taken using Micro plate reader at 540 nm. Blanks were the initial extract and sub fractions with buffer instead of enzymes and control were solvent in place of initial extract and sub fractions and enzyme. Acarbose at 1 mg/mL (in sodium phosphate buffer) was used as standard. Enzyme inhibition was calculated using the following equation:

$$\text{Inhibition}(\%) = \left(1\text{-}\frac{Abs\ S}{Abs\ E}\right)\text{x}100 \quad (4)$$

Abs S = Absorbance of Sample; Abs E = Absorbance of Enzyme

2.5.5. α-Glucosidase inhibitory assay

α-glucosidase enzyme inhibitory assay was performed by following the standard protocols from Apostolidis et al. (2007) with some modifications. In 96-well plate 50 μL of each sample (1 mg/mL) was added to 100 μL of (1 U/mL) α-glucosidase enzyme (Sigma-Aldrich) in 0.1 M potassium phosphate buffer (pH 6.9). The mixture was incubated at 25 °C for 10 min after which 50 μL of ρNDG was added at 5 s intervals and further incubated at 25 °C for 5 min. Readings were taken using Micro plate reader at 405 nm. Blanks were initial mother extract, sub-fractions with solvents instead of enzyme and control is enzyme and solvent in place of initial mother extract and sub fractions. Acarbose at 1 mg/mL (in sodium phosphate buffer) was used as standard. Enzyme inhibition was calculated using the following equation:

$$\text{Inhibition}(\%) = \left(1\text{-}\frac{Abs\ S}{Abs\ E}\right)\text{x}100 \quad (5)$$

Abs S = Absorbance of Sample; Abs E = Absorbance of Enzyme

2.5.6. Derivatization for GC Q-TOF MS

Derivatization was done for all 5 samples ($MeOH_i$, $Hexane_f$, $EtOAc_f$, $Acetone_f$ and $MeOH_f$) to improve peak determination and obtain explicit GC Q-TOF MS profiling. Samples were prepared following the method of Proestos and Komaitis (2013) with some modifications. 2 mL of the samples (in methanol) were taken in amber vials (Initially rinsed with toluene and methanol) and solvent evaporated off. After that 3 mL of ethyl acetate was added to each, vortexed and de-humidified with sodium sulphate. In reaction tubes, 100 μL of samples were added to 200 μL of BSTFA (kit, Supelco) and 50 μL of DMSO (to catalyse the reaction). The reaction media was vortexed and kept on water bath at 80 °C for 45 min.

2.5.7. GC Q-TOF MS determination

The derivatised samples were injected into a GCMS Agilent system of model 7200 accurate-mass GC Q-TOF MS connected to a 7890A GC system and the detector being quadropole time of flight (Q-TOF) mass spectrometer. The spectrometer was equipped with an Agilent J &W GC column of model- HP-5MS of dimension 30 m × 0.25 mm × 0.25 um. It was run following the conditions performed by Proestos and Komaitis (2013). The injector temperature was at 280 °C and detector at 290 °C. GC was done using splitless mode with 1 min splitless- time. The temperature programmed as follows: from 70 to 135 °C with a 2 °C/min, hold for 10 min, from 135–220 °C with 4 °C/min, hold for 10 min, from 220 to 270 °C with a 3.5 °C/min and then hold for 20 min. A post run of 10 min at 70 °C was seen to be sufficient for next injection. The carrier gas flow rate was maintained at 1.9 mL/min. Identification of compounds were done by matching the retention time of the peaks obtained with that of spectral data using Wiley and NIST libraries.

2.5.8. Statistical analysis

The TFC, TPC and DPPH assays were performed in triplicates and the results were expressed as means ± SD using Microsoft Excel. The enzyme analysis was performed in six replicates and evaluated by analysis of variance using one-way ANOVA followed by post hoc analysis using Tukey's post *t* test and Dunnett using IBM SPSS. A $p < 0.05$ value was regarded as significant.

3. Results and discussion

In this study, the α-amylase and α-glucosidase enzymes inhibition as well as extensive chemical profiling using GCMS (with QTOF) were performed for the first time for *M. pudica*.

3.1. Chemical class identification

The dried powder was tested for the presence of different and major classes of organic compounds like alkaloids, flavonoids, terpenoids, saponin, coumarine, anthrocyanin, carotenoids etc. The results are depicted in Table 1.

Phytochemical screenings for the different parts of *M. pudica* have already been reported for the presence of alkaloids, terpenoids, glycoproteins, crocetin dimethyl ester, phytosterol, glycosides, flavonoids, quinone, phenolic compounds, saponins, coumarins and tannins (Khare, 2004; Gandhiraja et al., 2009; Tamilarasi and Ananthi, 2012). Our analysis was found to be quite consistent and in agreement with the previously published reports on the same plant.

3.2. In-vitro analysis of TFC, TPC and DPPH

The initial extract ($MeOH_i$) and sub-fractions ($Hexane_f$, $EtOAc_f$, $Acetone_f$ and $MeOH_f$) were analysed for their total flavonoid and phenol contents as well as their free radical scavenging activity was evaluated through DPPH assay. The results are depicted in Table 2.

Extraction is an important step involved in the discovery of bioactive agents from medicinal plants. Different extraction methods have been employed to extract different types of phytoconstituents from plant materials. Biological activities of plant extracts have shown significant differences depending upon the different extraction methods, emphasizing the importance for the selection of the suitable extraction method with particular solvent (Hayouni et al., 2007). The current study performed initial extraction with methanol followed by subsequent fractionation using hexane to remove fatty acid, wax and similar materials. This was followed by ethyl acetate, acetone and finally with methanol. Various solvents extract out different classes of phytoconstituents based on their different polarity index. The ethyl acetate as a semi-polar solvent, acetone as a polar solvent and methanol as a very polar solvent have the tendency to successfully extract out most of the flavonoids and phenolic compounds during extraction and fractionation processes (Umar et al., 2010). Hence, they showed high TFC and TPC. However, methanol is the most polar solvent used in this study, consequently, methanol extract ($MeOH_i$) contained the most flavonoids in comparison to other fractions. Flavonoids and other phenolic compounds are main plant-derived bioactive-compounds and are known as natural antioxidants due to their redox properties, allowing them to act as free radical scavenger, lipid peroxidiser, hydrogen donors, reducing agents and metal ion chelators (Javanmardi et al., 2003). Total phenolic content assay determines the total phenolic type of compounds while TFC determines total flavonoid components in the sample. Calibration curve of gallic acid and quercetin standard for phenolic and flavonoid assays were plotted with r^2 as 0.998 and 0.999 respectively.

$MeOH_i$, the initial extract (mother extract), was found to have the lowest IC_{50} values (7.18 ± 0.0005) for DPPH free radical scavenging assay and the lower the IC_{50} value the stronger free radical scavenger it is (Nickavar et al., 2006). The subsequent sub-fractions were to have progressively higher IC_{50} values which are consistent as scavenging compounds had already been removed by the subsequent fractionations. Hexane fraction showed a lower TFC, TPC reading than the others and a higher IC_{50} for DPPH assay. Although showing a weaker antioxidative activity of 92.302 ± 0.0077 that may be attributed to the fraction showing the presence of the dodecaboranes in the GC results. As will be stated later in Section 3.4, that dodecaboranes may be the cause for the radical scavenging activity. This fact can also be attributed to hexane fraction supposedly contains mostly fatty acids, lower hydrocarbons and wax of the plant but some small phenolic acids may have been extracted that resulted in a slight high TPC content. Fatty acids have been reported to show free radical scavenging activity in some studies and may be the reason for showing some radical scavenging activity as the IC_{50} was found to be 92.302 ± 0.0077 μg/ mL (Hayouni et al., 2007).

Amongst the fractions, methanol fraction ($MeOH_f$) showed the highest value for IC_{50} for DPPH depicting that most of the free radicals scavenging compounds had already been fractionated out with ethyl acetate and acetone. Ethyl acetate ($EtOAc_f$) and acetone ($Acetone_f$) fractions were both found to be rich in phenolic acid and flavonoids contents comparatively. $EtOAc_f$ had a high TFC value of 3.90 mg/g equivalent to quercetin while $Acetone_f$ had a higher phenolic content of 60.07 mg/g equivalent to gallic acid. Methanol initial extract ($MeOH_i$) was seen to have the lowest IC_{50} value, highest amount of TFC and TPC. Amongst the fractions, comparatively $EtOAc_f$ and $Acetone_f$ showed commendable high results.

Our results were found to be in agreement, however, with a slightly higher content of TFC and TPC in comparison to the similar previous studies done on *M. pudica* evaluating the DPPH, TFC and TPC (Suganthi et al., 2011; Zhang et al., 2011).

3.3. α-amylase and α-glucosidase enzyme inhibition assay

Plants with traditional claims in the management of diabetes have been studied earlier and particularly three such anti-diabetic studies for *M. pudica* have already been performed (Umamaheswari & Prince, 2007; Sutar et al., 2009; Manosroi et al., 2011). A particular therapeutic agent or plant extract works following one or more mechanism of which post prandial hyperglycaemic enzyme inhibition is quite notable (Shim et al., 2003). The enzymes α-amylase and α-glucosidase are related to post prandial high blood glucose levels (BGL). α-amylase is connected to breaking the polysaccharides into disaccharides and oligosaccharides. α-glucosidase works on the disaccharides and polysaccharides to break them into glucose monomers aiding carbohydrate digestion. Inhibition of these enzymes can lead to a control on post-prandial BGL by controlling carbohydrate digestion and hence controls diabetes significantly (Oboh et al., 2012; Apostolidis et al., 2007; Yao et al., 2013). Enzyme inhibition assay for plant's extract determines the inhibitory capacity of the said sample against the enzymes and it is one of the mechanisms through which a plant could show its anti-diabetic activity. This is the first study on *M. pudica* for their role as a digestive enzymes inhibitor. The results of α-amylase and α-glucosidase enzymes inhibition assays are shown in Table 3.

Table 2
DPPH, TPC, and TFC results.

Extract/ Fractions	DPPH, IC_{50} μg/mL	TPC mg/g	TFC mg/g
MeOHi	**7.18 ± 0.0005**	**57.431 ± 1.096**	**16.97 ± 1.472**
$Hexane_f$	92.302 ± 0.0077	28.523 ± 5.296	0.927 ± 0.461
$EtOAc_f$	49.59 ± 0.0024	42.550 ± 2.228	3.90 ± 0.059
$Acetone_f$	45.63 ± 0.0012	60.07 ± 1.066	3.144 ± 0.2112
$MeOH_f$	158.4 ± 0.0004	27.45 ± 2.083	4.692 ± 0.313

Table 3
α- amylase and α- glucosidase inhibition assay.

Extract, Fractions (1 mg/mL)	α- amylase (% inhibition)	α- glucosidase (% inhibition)
$MeOH_i$	**33.86 ± 5.599**	**95.65 ± 0.911***
$Hexane_f$	−10.583 ± 10.246	0.884 ± 2.617
$EtOAc_f$	**18.65 ± 6.837**	**51.87 ± 3.106***
$Acetone_f$	15.64 ± 5.55	16.04 ± 4.04*
$MeOH_f$	27.21 ± 5.816	4.83 ± 2.373*
Acarbose (std)	**28.24 ± 13.664**	**36.93 ± 2.701***

* The results were significantly different ($p < 0.05$).

All samples were done in sets of six and standard deviation was calculated of best four. The initial methanol extract ($MeOH_i$), subsequent sub-fractions and acarbose were all tested at 1 mg/mL concentration. We took α-glucosidase as our focal point since α-amylase was found to show some anomaly and incongruity during analysis. In conjunction to previous results, the initial methanol extract ($MeOH_i$) was found to show a promising inhibitory effect on both the enzymes especially showed significant results for α-glucosidase (95.65%) while hexane fraction did not show inhibitory activity against both the enzymes. This could be due to the fact that hexane fraction does not possess anti-diabetic compounds which are present in other fractions revealing inhibitory activity against both the digestive enzymes. Amongst the fractions, $EtOAc_f$ showed comparatively good inhibitory activity with 51.87% inhibition for α-glucosidase and 18.65% inhibition for α-amylase, respectively. The results were compared to acarbose (standard digestive enzymes inhibitor) of same concentrations 1 mg/mL. $MeOH_f$ (methanol fraction) showed slightly higher or comparable inhibition than acarbose for α-glucosidase, however, $MeOH_i$ (mother extract) showed almost 3 times more inhibition than acarbose for α-glucosidase. Amongst the other sub-fractions, $EtOAc_f$ showed 2 fold more activity than acarbose of same concentration. Results for α-amylase, however, were found to be insignificant and incongruent but the α-glucosidase results were found to be significant ($p < 0.05$) after ANOVA analysis and this complied with our experimental outcomes since amylase was observed to show some instability and incongruity during analysis. A sample could also be verified for its enzyme inhibitory potential based on α-glucosidase activity singularly (Mai et al., 2007). In congruent to that, $MeOH_i$ and $EtOAc_f$ showed three and two fold inhibitory activity ($p < 0.05$) against α-glucosidase enzyme which could prove to be a commendable future anti-diabetic agent. The good inhibitory activity of the sample may be attributed for the presence of high amount of flavonoid and phenolic contents in *M. pudica*. Flavonoids have already been proved and reported to exert enzyme inhibitory action, thereby, helps in the regulation of glucose absorption and consequently glucose homeostasis (Tadera et al., 2006; Pereira et al., 2011).

3.4. GC Q-TOF MS results

GCMS is extensively employed for chemical profiling of biological systems, plant extracts and fractions, essential oil, fatty acids, bioactives etc. Its use in phenolic acid, polyphenolic compounds and flavonoids detection is still a new prospect due to their high molecular weight and high vaporizing points which make detection for such compounds quite difficult. However, to overcome this cumbersome problem, derivetisation is done to reduce the polarity and makes the compounds more prone to fragmentation. In this study, N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA)was used to accomplish derivetisation by carefully following the steps to ensure minimum light and moisture exposure. The samples were analysed by the state of art Gas Chromatography Time of Flight Mass Spectra (GC Q-TOF MS) for identification of the chemical compounds and were matched to the NIST library. The results are shown in Table 4 for the major compounds, their PubChem and ChemSpider identification reference along with structures and pharmacological activity.

Fig. 1 shows the number of compounds that were detected based on the MS peaks. $Hexane_f$, $EtOAC_f$, $Acetone_f$, $MeOH_f$ and $MeOH_i$ (extract) were found to contain 34, 22, 33, 29 and 46 different compounds, respectively. It has already been discussed that *M. pudica* has been reported to have different classes of organic compounds like alkaloids, flavonoids, saponins, quinone and phenols. Consistent to the classes, phenols like benzeethanol, di-phenols, quinone & quinolinone, alkaloids like benzeneamide, iminocarbonitrile etc have been identified in this study. Hexane is a non-polar solvent and it was used to bring out the fatty acid and wax components which are basically long chain hydrocarbons from the initial methanol extract (Harborne, 1984). Hexane fraction found to contain a plethora of fatty acids and long chain hydrocarbons but also interesting compounds like carotene, dioxolane, methyl sulphide and acids like malonic, aspartic and the newly identified mannopyranosyl dodecaborane. Existence of borane is being reported for the 1st time in this plant through GCQ-TOF MS technique. Dodecaboranes have recently gained much exposure for being a very strong free radical scavenger through the boron neutron capture theory (BNCT) and has been reported to show strong anticancer activity with low toxicity (Valliant et al., 2012). The presence of dodecaboranes could be a reason for *M. pudica*'s strong scavenging activity as seen in the extract ($MeOH_i$). Literatures review on the anti-diabetic activity of dodecaboranes didn't result any study hence, they are stipulated to being strong candidates for future prospective study for their role as anti-diabetic agents. The $EtOAC_f$, and $Acetone_f$ showed good enzyme inhibition which can be designated due to a vast range of free radicals scavenging compounds found in both fractions. An alkaloid compound (see Table 4) of a very high molecular weight was identified in the $EtOAC_f$.

In $Acetone_f$, apart from the dodecaborane, compounds like *p*-quinone, dioxime, benzothiazole, tyrosol etc were also identified as shown in Table 4. $MeOH_f$ showed a lower activity in enzyme inhibition which can be attributed to the fact that it was the last fraction to be derived so most of the anti-diabetic components had already been successfully fractionated into the $EtOAC_f$ and $Acetone_f$. Although, comparatively it showed pretty much similar compounds like its counter fractions some interesting compounds were also identified like fluoro-anisidine, quinolinone, and a very high molecular weight compound with the MF $C_{27}H_{38}O_4S_2$. And lastly, the $MeOH_i$ which was the mother extract was analysed and apart from the compounds found in the fractions, it showed some other compounds too which were not detected, this could be the fact that the initial extract underwent continuous heat under reflux for the fractionation procedure which may be responsible for the degradation of some key compounds. The identified compounds were iminocarbonitrile, dioxolane, eicosatriynoic acid and glucopyranosiduronic acid. Some compounds were unidentified whose retention time and molecular weights are tabulated in Table 4. These could be new compounds or the ones which were not derivetised enough to identify through GC Q-TOF MS analyses, but in both cases the chances are more towards them being new compounds. Glycine was found to be present in all the samples as one of the major constituents.

$Hexane_f$ despite being basically a non-polar fraction revealed good radical scavenging activity and phenolic content (28.523 mg/g) which might be attributed to the carboxylic and phenolic acids or their derivatives, carotenes, sulphur containing compounds and simple phenols in the hexane fraction. The presence of docedaboranes could be attributed to hexane's scavenging power.

The ethyl acetate is a semi-polar solvent with low toxicity and is commonly used for polyphenolic compound extraction due to its particular affinity for mostly phenolic compounds (Oboh et al., 2012). $EtOAc_f$ showed 18.65% and 51.87% inhibition for α-amylase and α-glucosidase enzymes, respectively while a TPC was found to be 42.550 mg/g. GC profiling revealed phenols like benzeneethanol, phenyl ethanol, benzylglycerol, and acids like aspartic, carbamic and butanoic which may be responsible for the activities (Bhandari and Kawabata, 2004). Two unknown compounds were also detected which could be flavonoid or any other components which might be responsible for the high flavonoid content and good anti-diabetic activity in ethyl acetate fraction as flavonoids were not singularly detected by the GC Q-TOF MS. Basically the two fold inhibitory activity could be manifested due to the presence of flavonoid, glycoside type of compounds which were not successfully detected by the GC Q-TOF MS in this study. Hence, it might be suggested to undergo extensive isolation for finding the possible anti-diabetic compounds responsible for such potent activity.

$Acetone_f$ showed moderate enzyme inhibitory activity (15.64% & 16.04%) against α-amylase and α-glucosidase enzymes as well as high TPC (60.07 mg/g) and modest TFC (3.144 mg/g). Compounds like glycine, tyrosol, benzothiazole, dodecaboranes, butanoic acid, benzyloxypropanoic acid, carbamic acid, tyrophanamide and quinine were identified in acetone fraction. The small molecular weight organic acids and

Table 4
GCMS chemical profiling with PubChem references and pharmacological activities.

Compounds and chemical reference	Retention time (RT)	Activity/PubChem or Chemspider, reference no
Hexane$_f$		
Glycine,(Ntrifluoroacet) (derv.)*	125.98476	CID-750; Non-essential amino acid, Glycine agent, for fluid irrigation, Apoptotic activity, (PMID-1960447; (Zhong et al., 2003)
1,2-diethyl-3,4-dimethyl benzene	162.0158	CID-590292; Disinfectant, anti-infective agent, component of Bayer's process (Smeulders et al., 2001)
Carotene (derv.)	171.966	Anti-oxidant, Cancer fighter (Omenn et al., 1996)
2-tert-butyl-2-phenyl- 1,3-Dioxolane	149.01037	CID-00590932
2-Hydroxyethyl-methyl Sulfide (as ester)*	149.01063	ChemSpider-71259
Glycolic acid (as ester)	133.0355	CID-757; Keratolytic agent, Acne treatment, anti-infective, Bioactive (Murad et al., 1995)
4-(2-phenylethyl)-phenol	195.9727	CID-80630; Oestrogen mimetic, bioactive(Buckingham & Macdonald 1996)
Monoamidomalonic acid (as ester)	229.005	CID-9601826. Radical scavenger (Poyrazoglu et al., 2002)
2,2'-Ethylenediphenol	214.00715	CID-572469 Potent anti-oxidant, radical scavenger(Buckingham & Macdonald, 1996)
2-Deoxy-D-ribose, tris(trimethylsilyl) ether, methyloxime (derv.)	322.97158	Chemspider-7876007
I-Tyrophanamide	163.0235	CID-595943
2-hydroxy-benzenethanol	185.97935	CID-82200
1-(D-Mannopyranos-1-yl)-1,7-dicarba-closo-dodecaborane(12)		Dodecaborane reported 1st time for this plant.
EtOAc$_f$		
N-Aspartic acid (derv.)	184.9719	CID-586809; Radical scavenger (Noda et al., 2003)
1-phenyl-2-(2-cyclopropenyl)-ethanol (derv.)	62.9874	Phenol, anti-oxidant (Kukula-Koch et al., 2013)
(R)-(+)-1- Benzylglycerol	106.9697	CID-98072; Bioactive, anti-cancer/ tumor Anti-oxidant (Mukai et al., 1989)
N-Benzoyl-dl-3,4-dehydroprolylglycine	171.96577	ChEMBL-858271; Show inhibitory activity against Dengue, viral inhibitor (recently found)
4-biphenyl carbamic acid (derv.)	170.00456	Free radical scavenger, CNS active agent (Bhandari & Kawabata, 2004)
2-hydroxy-benzeneethanol	185.9794	CID-6054; Antibacterial, Bioactive (Jinshun et al., 2009)
2-amino-3-oxo-butanoic acid (derv.)	184.9719	CID-219; radical scavenger (Bhandari & Kawabata, 2004)
Acetone$_f$		
2,5- Cyclohexadiene-1,4-dione, dioxime or p-quinone	184.97255	CID-7737; radical scavenger, Highly active agent (Lee et al., 1998)
4-hydroxy-benzeenethanamine, Tyramine (derv)	106.96997	CID-5610; Anti-microbial agent, CVS active, anti-oxidant (Cai et al., 2012)
2-methyl-benzothiazole	149.01049	CID-8446; Antimicrobial and anti- mycotic (Sigmundová et al., 2008)
2-benzyloxy-propanoic acid	140.99371	CID-245987; Derivative of this show anti-diabetic effect (AID-188221) (Hulin, 1997)
4-hydroxy-benzeneethanol (Tyrosol)	137.983	CID-10393; Anti-oxidant, anti-cancer, CNS acting agent (Miro-Casas et al., 2003)
MeOH$_f$		
Acetic acid (large molecule)	125.93158	Antibacterial agent and reducing agent. (Buckingham & Macdonald, 1996)
3-Fluoro-ρ-anisidine	140.99374	CID-581110; Protein kinase inhibitor, radical scavenger (Paul et al., 1998)
3-acetyl-4-hydroxy-2(1H)-quinolinone,	202.96304	CID-117739; Tautomer, bioactive with high radical scavenging activity, antibacterial, anti-fungal, analgesic, CVS active agent, HIV inhibitor (Dayam et al., 2006; (Abdou, In Press).
2,4-Dithiapentane	150.98977	CID-15380; Antimicrobial, Muscarinic receptor antagonist, anxiolytic, used in parkinson's, anti-cholinesterase poisoning. (Olorunnisola et al., 2012)
MeOH$_i$		
9-Imino-12-phenyl-10,11-dioxa-tricyclo[6.2.2.01,6]dodecane-7,7,8-tricarbonitrile	206.99234	CID-387810; active against tumor cell line, anti-oxidant (Grever et al., 1992)
4-Phenylbutan-2-ol, tert-butyldimethylsilyl ether (derv.)	206.99217	CID-61302; Anti-hypertensive agent (Allegretti et al., 2013)
2-Oxa-3-azabicyclo[4.4.0]dec-3-ene, 5-methyl-1-trimethylsilyloxy-, N-oxide (derv.)	214.0093	CID-554483
1-Naphthalenecarboxylic acid	184.97375	CID-6847; radical scavenger(Bhandari & Kawabata, 2004)
5,8,11-Eicosatriynoic acid, tert-butyldimethylsilyl ester (derv.)	163.0257	CAS-13488-22-7; Lipo-oxygenaseinhibitor used in kidney Ca^{2+}mobilisation.(Bezakova et al., 1996) PMID:11169157
β-D-Glucopyranosiduronic acid, (methyl ester)	171.96792	CID-553478 (Ghosh et al., 2014)

* The derivatised compounds are pointed as (derv.) and (ester). Repetitive compounds not included.

phenolic acids have been claimed to be a good source of free radical scavenging activity (Loliger, 1991; Poyrazoglu et al., 2002). Compounds having free radical scavenging activities have been in turn related to possessing inhibitory activity on digestive enzymes that can be termed as anti-diabetic in nature (Matsui et al., 2001).

$MeOH_f$ showed a weak enzyme inhibition towards both enzymes which could be attributed to the fact that most of the flavonoids and phenolic compounds had already been successfully fractionated into the $EtOAC_f$ and $Acetone_f$. Moreover, 3-acetyl-4-hydroxy-2(1H)-quinolinone of particular interest was identified that has already been reported as a potent radical scavenger with anti-cancer, anti-fungal, analgesic and cardiovascular (CVS) agent (Abdou, in press). Also, 3-fluoro-ρ-anisidine which is a protein kinase inhibitor has been identified which is a strong antioxidant (Paul et al., 1998). Both of these compounds are powerful antioxidants with additional benefits of anti-microbial activity.

The initial methanolic extract ($MeOH_i$) showed maximum number of compounds and some were larger and important molecules like imino-tricarbonitrile, phenylbutanol, napthalein carboxylic acid, eicosatriynoic acid, β-D-glucopyranosiduronic acid and five unidentified compounds amongst the 46 compounds detected through GC Q-TOF MS analyses. The $MeOH_i$ showed a very strong α-glucosidase activity (3 times than acarbose of same concentration, 1 mg/mL) with nearly 4 times more flavonoid content than its counterparts which could be the reason for a 95.65% α-glucosidase and 33.86% amylase inhibition. Compounds that show strong free radical scavenging activity and anti-oxidative capacity have been strongly co-related to combating hyperglycaemia (Matsui et al., 2001). Such compounds are usually organic acids like hydroxybenzoic, acetic,

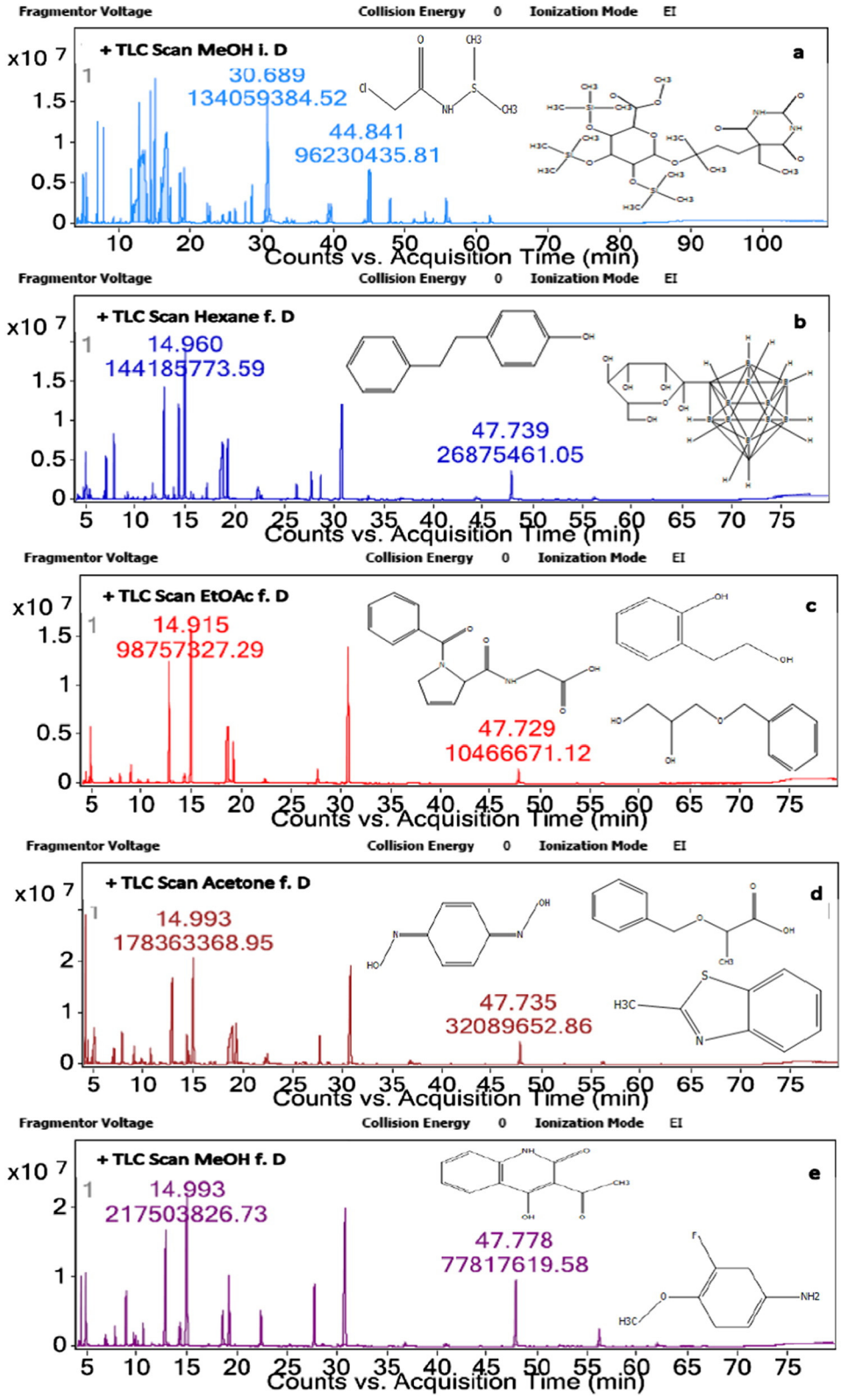

Fig. 1. (a) TIC scan of initial methanol extract ($MeOH_i$), (b) TIC scan of hexane fraction($Hexane_f$) from $MeOH_i$, (c) TIC scan of ethyl acetate fraction ($EtOAc_f$) from $MeOH_i$, (d) TIC scan of acetone fraction ($Acetone_f$) from $MeOH_i$ and (e) TIC scan of methanol fraction(MeOHfr) from $MeOH_i$.

phenyl acetic, benzoic acid, simple phenols, phenolic acid, polyphenolic compounds, various classes of flavonoids and glycosides. Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarines, flavonoids, hydrolyzable and condensed tannins, lignans, and lignins. Such types of compounds have been abundantly identified in *M. pudica* (Bezakova et al., 1996; Grycova et al., 2007; Zadernowski et al., 2009; Wang et al., 2012; Blainsky et al., 2013; Djeridane et al., 2013; Kukula-Koch et al., 2013; Yao et al., 2013).

The principle function of antioxidants is to delay the oxidation process of oxidizing molecules by either inhibiting the initiation or chain

propagation of those molecules by the free radicals which are continuously produced in our body due to various biochemical mechanisms (Namiki, 1990). The GC Q-TOF MS analyses by far couldn't identify flavonoids, phenolic compounds and glycosides of high molecular weights that are usually projected and linked to anti-diabetic activity as described before. This limitation is suggestive of another study for the isolation of principle agents through advanced analytical techniques i.e., HPLC, silica gel or sephadex LH 20 column chromatography etc.

4. Conclusion

The present study delved deeper into a probable anti-diabetic mechanism of this plant showing anti-diabetic activity by performing α-amylase and α-glucosidase enzyme inhibition assay as well as performing chemical profiling using GC Q-TOF MS analyses. We have reported positive activity of the initial extract ($MeOH_i$) and fraction ($EtOAc_f$) against the digestive enzymes especially α-glucosidase as of three- and two-fold as compared to acarbose at the same concentration. *In-vitro* antioxidant assays (DPPH radical scavenging assay) along with TFC and TPC have been performed. The radical scavenging activity coupled with the TFC and TPC assay showed high amount of free radical scavengers in the form of flavonoids and phenolic compounds present in *M. pudica* that have been attributed to anti-diabetic activity. In conjunction with the three *in vitro* assays, the α-amylase and α-glucosidase enzyme inhibition assays showed potent inhibitory activity (two- and three-fold inhibitory activity) as compared to acarbose at the same concentration with significant ($p < 0.05$) result. Chemical profiling has shown an abundance of phenolic compounds, organic acids, dodecaborane, quinine, quinolones etc. The dodecaborane is being reported for the first time in this plant. This plant is a neglected weed growing in dumps and wastelands with traditional anti-diabetic claims. This study has proven a strong anti-diabetic activity and promising compounds which can lead to future studies with respect to get new anti-diabetic agents for *M. pudica*. This weed could be proposed as a medicinal alternative to concurrent medicinal flora which could mean a cheaper source of herbal therapeutics. The chemical profiling didn't identify any deleterious or toxic compounds which could prove to be harmful. Hence, it is concluded that this weed is an excellent antioxidant herb which could be effective against diabetes mellitus.

Declaration of Conflict

None to declare.

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