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Optimization of extraction protocol for isolation of biomarkers with known anti-diabetic potential from fruits of *Tribulus terrestris* L

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Abstract
Simple reproducible extraction methodology has been optimized for the simultaneous extraction of secondary metabolites which are known to have anti-diabetic potential from the methanolic extract of fruits of plant *Tribulus terrestris* L. The influence of different solvent mixtures, solvent amounts, temperature, extraction time, and procedures for defatting on yield and profile of various classes of secondary metabolites was investigated. The compromise extraction solvent for all of the examined compounds is 20: 80 mixture of 2 N HCl and methanol, mixed in ratio 30:1 with plant material. The mixture was refluxed at controlled 80°C on a boiling water bath for about 6 hrs, filtered, extracted with chloroform and washed with alkali and distilled water. The extract was concentrated to dryness by evaporating the solvent at reduced pressure on Rotavapor buchi at 60° C and stored in the dark, on 20°C. Extraction efficiency and presence of markers was monitored and confirmed by Biochemical test and LCMS. Proposed extraction method can be used as an analytical tool for quality evaluation of plants and formulations containing *Tribulus terrestris* L.

Keywords: *Tribulus terrestris* L., anti-diabetic, solvent mixture, refluxed and LCMS

1. Introduction
Tribulus terrestris L. is a valuable herb known for its application in the folk medicine in various parts of the world. This plant is extremely rich in substances having potential biological significance, including: saponins, flavonoids, alkaloids, and other nutrients [1, 2]. It is extremely efficacious in most of the urinary tract disorders because it promotes the flow of urine, cools and soothes the membranes of the urinary tract, and aids in the expulsion of urinary stones and gout. It also stops bleeding from the tract and rejuvenates the urogenital system, both in males as well as females. *Tribulus terrestris* L. effectively controls the bleeding, in large doses, it imparts the laxative action, hence is used as an adjunct in the treatment of piles. It is commonly used in treating diabetes, urinary calculi, dysuria, gout and sexual debility [3, 4].
Phytochemical processing of raw plant materials is essentially required to optimize the concentration of known constituents and also to maintain their activities [5]. Extraction is an important step in the itinerary of phytochemical processing for the discovery of bioactive constituents from plant materials. Selection of a suitable extraction technique is also important for the standardization of herbal products as it is utilized in the removal of desirable soluble constituents, leaving out those not required with the aid of the solvents [6].

Fig 1: *Tribulus terrestris* L. and Its fruit with fruit powder

A wide range of technologies with different methods of extraction such as solvent extraction, maceration, decoction supercritical fluid extraction, microwave-assisted extraction and solid phase extraction are available nowadays [6, 7]. Hence, this review aim to describe and compare the most commonly used methods based on their principle, strength and limitation to help evaluating the suitability and economic feasibility of the methods [6, 8].

Extraction of the active principles is an essential step in evaluation of their bioactivity and chemical characterization. However, non-optimized extraction conditions can lead to non-reproducible results, losses, degradation and modification of the biomolecules. Inaccurate analysis can result in invalid conclusions about the chemical composition and the amount of secondary metabolites present in plant. Therefore, optimization of extraction conditions is important for maximizing yields of the compounds of interest, while minimizing the extraction of unwanted compounds [6, 9]. Refluxed solvent extraction is widely used technique suitable for extractions of small amounts of plant material in laboratory.

Many forms of raw plant material and herbal drugs derived from *Tribulus terrestris* L. are distributed in herbal market; however, the content of bioactive components in these products have not necessarily been quality-controlled [10]. Therefore, a simple, low-cost, and rapid extraction method for screening and quantitating bioactive components is strongly desired [11]. The objective of the present research work was extraction of the small molecular weight components specifically Diosgenin, Oleic acid, Harmine and Kaempferol, which are very well known for having anti-diabetic activity. Harmine regulates the expression of peroxisome proliferator-activated receptor gamma (PPARγ), the molecular target of the thiazolidinedione antidiabetic drugs, through inhibition of the Wnt signaling pathway [12]. Diosgenin can be useful, for instance, in blood and cerebral disorders, allergic diseases, diabetes and obesity [13, 14]. Kaempferol ameliorates hyperglycemia by improving insulin-stimulated glucose uptake in adipocytes and beneficial role in diabetes by preventing oxidative damage in pancreatic beta cells [15, 16]. Central Administration of Oleic Acid Inhibits Glucose Production and Food Intake [10]. In this paper, we investigated the influence of different solvent mixtures, solvent amounts, temperature, extraction time, techniques and procedures on yield and profile of various classes of secondary metabolites from fruits of *Tribulus terrestris* L. Extraction efficiency was monitored Biochemical test, LCMS/MS [17, 18].

Materials and Method

Collection of plant

Tribulus terrestris L. were collected from Padadhari, around 30 km away from Rajkot, Gujarat, India in the month of December and it was authenticated with specimen No. 10291(2) of H. Santapau at 'Blatter Herbarium' in St. Xavier's college, Mumbai-400001

Preparation of plant material

The fruits were washed thoroughly with tap water. The fruits were dried initially using tissue paper to remove excess of water and later were air dried thoroughly under shade at room temperature to avoid direct loss of phytoconstituents from sunlight. The shade dried material was powdered using grinder and sieved through an ASTM 80 mesh. It was then homogenized to fine powder and stored in an air-tight container for further analysis [10, 19].

Reagents and Standards

All chemicals and solvents used were of analytical grade and purchased from Merck (Darmstadt, Germany). Analytical standards Diosgenin, Oleic acid, Harmine and Kaempferol were procured from Sigma-Aldrich (Bengaluru, India).

Experimental

Optimization of extracting solvent

In this experiment, seven portion of 2.0 g leaf powder of *Tribulus terrestris* L. was transferred into seven different stoppered conical flasks containing 50 mL each of water, water: methanol (20:80), 2 N HCl : methanol (20:80), methanol, ethyl acetate, chloroform and n-hexane separately. The contents were mixed and kept in water bath at 80° C for 4.0 hr. These extracts were filtered through Whatman filter paper no. 41 and concentrated to dryness by evaporating the solvent at reduced pressure in pre-weighed, dried Rota flask on Rotavapor buchi at 60 °C The dried residue was weighed and the percentage extraction was calculated.

$$\% \text{ extraction} = \frac{M_{extract}}{M_{Herb}} \times 100$$

Where $M_{extract}$ is the crude extract mass (gm) and M_{herb} is the extracted herb mass (gm).

Optimization of extracting method

In this experiment, three portion of 2.0 g leaf powder of *Tribulus terrestris* L. was transferred into three different stoppered conical flasks containing 50 mL each of water: methanol (20:80), 2 N HCL: methanol (20:80), methanol and ethyl acetate separately. The contents were mixed and kept in sonicator for 8.0 hrs. Three portion of 2.0 g leaf powder of *Tribulus terrestris* L. was transferred into three different stoppered conical flasks containing 50 mL each of water: methanol (20:80), 2 N HCL: methanol (20:80), methanol and ethyl acetate separately. The contents were mixed and kept orbital shaker at 500 rpm for 8.0 hr. Three portion of 2.0 g leaf powder of *Tribulus terrestris* L. was transferred into three different three neck round bottom flasks containing 50 mL each of water: methanol (20:80), 2 N HCL: methanol (20:80), methanol and ethyl acetate separately. The contents were mixed and refluxed at 80 °C for 8.0 hr. These extracts were filtered through Whatman filter paper no. 41 and concentrated to dryness by evaporating the solvent at reduced pressure in pre-weighed, dried Rota flask on Rotavapor buchi at 60 °C. The dried residue was weighed and the percentage extraction was calculated.

Qualitative phytochemical screening analysis

Qualitative Phytochemical screening analysis of the T. terrestris extract was performed to identify the bioactive constituents; this study was carried out by using standard procedures, plant extracts were detected by the following tests [19, 20].

Detection of alkaloids

1 ml of extract was taken and added with a few ml of dilute HCl and filtrate the sample. The alkaloid reagent of Mayer's Test (Potassium Mercuric Iodine Solution) was carefully added to the test solution. Formation of creamy white precipitate indicates presence of alkaloids.

Detection of Saponins

1 ml of extract was dissolved with 2ml of distilled water. The

suspension is shaken well for few minutes. A layer of foam indicates the presence of Saponins.

Detection of flavonoids
Sodium Hydroxide Test 2 mg of extract was dissolved in 2ml of distilled water; 2ml of 10% sodium hydroxide solution was added to the test sample aqueous basic lead acetate solution was added. Formation of yellow precipitate indicates the presence of flavonoids [21, 22].

Detection of free fatty acid
Take 1ml of chloroform and add a methanol and one drop of oil. To this add 1drop of iodine. Chloroform dissolve sample give red colour which decolorizes the iodine giving brown colour. This indicates the presence of fatty acids [22].

Optimization of volume of extraction solvent
Optimization for the volume of the solvent for the extraction was carried out by keeping constant the weight of the plant powder (5.0 g) and time of refluxing i.e. 6hrs (Equilibration time). Initially volume was fixed at 50 mL and an increment of 50 mL in the subsequent experiment. The volume was varied till the weight of extract obtained was constant

Optimization of time of extraction solvent
Time was determined by using the optimized volume with single extraction and keeping the weight of the plant powder constant at 5.0 g, but refluxing the contents of the flask at varying time intervals i.e. at 4 hrs, 6 hrs, 8 hrs, 10 hrs and 12 hrs. The time after which there was no further increase observed in the weight of the extract was selected as the optimized time.

Selection of non-polar solvent and removal of unwanted compounds
To enhance concentration of desired phytochemicals and to get rid of polar waters soluble matter, material extracted by refluxing at 80 °C for 8.0 hrs with 2 N HCl: methanol (20:80), was re-extracted using 50 ml of each solvents like n-hexane, chloroform, toluene and Diethyl ether in separating funnel twice and rinsed thrice times with 2 N NaOH and then rinsed thrice with distilled water. The extract was then passed through a filter bed of Na_2SO_4 to eliminate any remaining water. The samples were concentrated to dryness by evaporating the solvent at reduced pressure on Rotavapor buchi at 60 °C. The dried residue was weighed and the percentage extraction was calculated.

Optimization of numbers of non-polar solvent extraction
The number of extractions was determined by using the optimized volume of the solvent and optimized time of extraction and keeping the weight of the leaf powder at 5.0 g but re-extracting the same weighed powder, over and over again and calculating the weight of the extract after each extraction. The number of extraction after which there was no further increase in the weight of the extract was considered as the optimized number of extraction.

LCMS (Liquid chromatography- mass spectroscopy) analysis
Extracts were diluted with mobile phase premixed in 1:1 ratio, to obtain a final concentration of 50 mg/mL. The Agilent 1100 series HPLC system and 3200 Q Trap LCMS/MS system of Applied Biosystem MDS/SCIEX was used for the analysis. The optimized conditions [Mobile phase A: 0.01 M Ammonium Acetate buffer; pH 7.5 adjusted with ammonia And Mobile phase B: Acetonitrile: Methanol (80:20)] gradient elution system on a C8 reversed-phase column with a flow rate of 1 mL/min. Extract were scanned at both positive mode (Q1MS +ve) and negative mode (Q1MS –ve).

Results and Discussion

Optimization of extracting solvent
The dried residue was weighed and the percentage extraction was calculated. The results are shown in table 1. and% extraction against various solvent mixtures is plotted in Figure 1.

Table 1: Optimization of extractive value

Obs. No.	Solvent	M_{herb} (gm)	% Extraction
1	Water	2.0256	6.67
2	Water : methanol (20:80)	2.0856	14.33
3	2 N HCl : methanol (20:80)	2.0775	14.53
4	Methanol	2.0056	13.09
5	Ethyl acetate	2.0741	12.88
6	Chloroform	2.0568	9.46
7	n-Hexane	2.0074	4.32

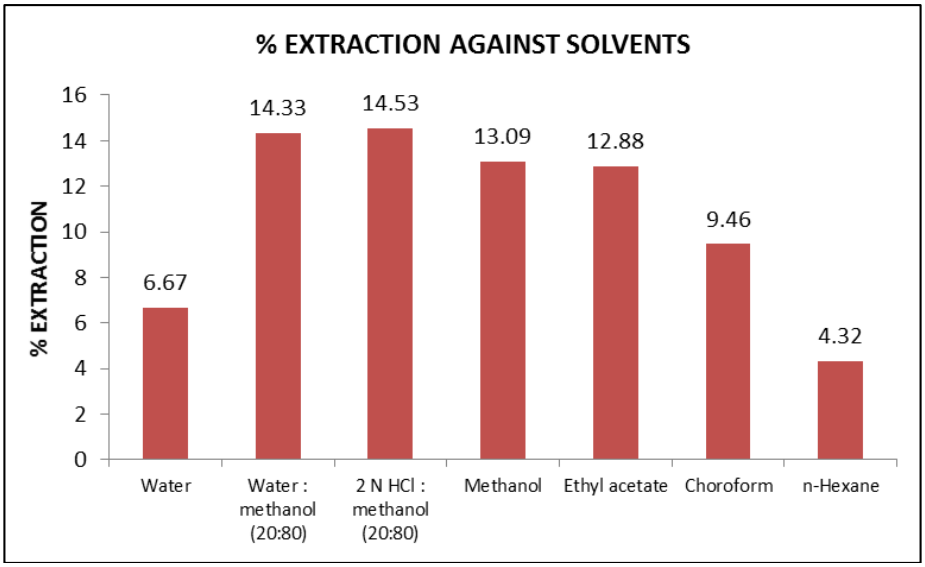

Fig 2: Optimization of extractive value against solvent mixtures

Optimization of extraction method

All three methods were studied against four solvent mixtures such as water: methanol (20:80), methanol 2 N HCl: methanol (20:80) and ethyl acetate. The results are shown in table 2 and % extraction against various solvent mixtures is plotted in Figure 3. Qualitative Phytochemical screening analysis of each the T. terrestris extract which were extracted by Reflux at 80 °C for 8 hrs is tabulated in table 3.

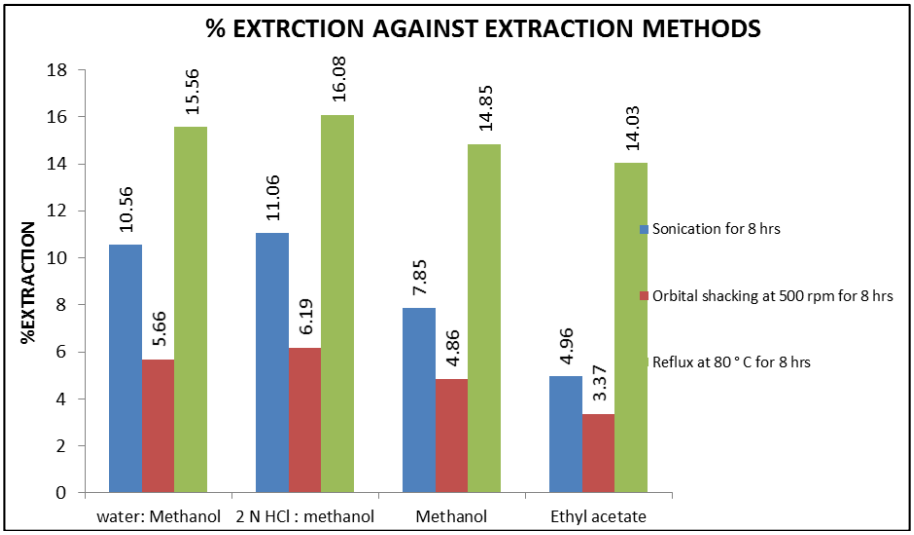

Fig 3: Optimization of extraction with various extraction methods and solvent mix

Table 2: Optimization of extraction method

Obs. No.	extracting method	Solvent	M_{herb} (gm)	% Extraction
1	Sonication for 8 hrs	Water : methanol	2.0236	10.56
		2 N HCl: methanol	2.0014	11.06
		Methanol	2.0045	7.85
		Ethyl acetate	2.0145	4.96
2	Orbital shacking at 500 rpm for 8 hrs	Water : methanol	2.0366	5.66
		2 N HCl: methanol	2.0844	6.19
		Methanol	2.0525	4.86
		Ethyl acetate	2.0684	3.37
3	Reflux at 80° C for 8 hrs	Water : methanol	2.0010	15.56
		2 N HCl :methanol	2.0149	16.08
		Methanol	2.0362	14.85
		Ethyl acetate	2.0355	14.03

Table 3: Qualitative data of biomarkers in various solvent mixtures

Obs. No.	Solvent	Alkaloid	Saponin	Flavonoid	Fatty acid
1	Water : methanol	-	+	+	++
2	2 N HCl : methanol	+	++	+	++
3	Methanol	-	+	+	++
4	Ethyl acetate	-	-	+	++

+ Weakly positive (presence), ++strongly positive (presence), - Negative (absence)

Optimization of time of extraction solvent

The refluxing time in minutes and the corresponding percentage extraction is shown in Table 4.

Table 4: Optimization of Time

Obs. No.	Weight in gm	Time in hrs	% Extraction
1	5.0145	2	4.34
2	5.0035	4	8.22
3	5.0852	6	12.76
4	5.0110	8	16.67
5	5.6325	10	16.57
6	5.0023	12	16.66
7	5.0202	18	16.35

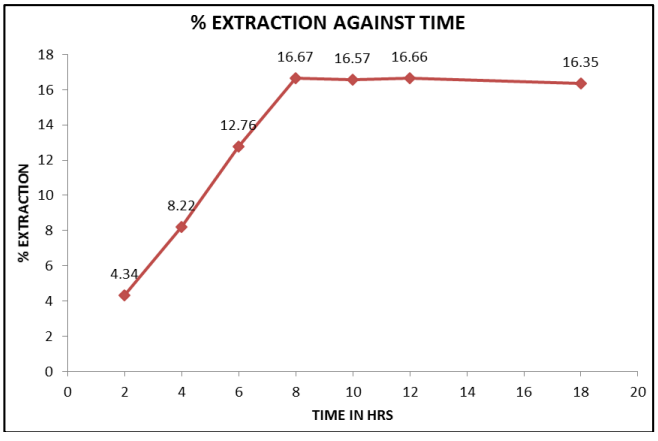

Fig 4: Optimization of Time for extraction

Selection of non-polar solvent and number of extraction

All 4 selected solvents were analysed for extraction. Results were plotted in figure 5.

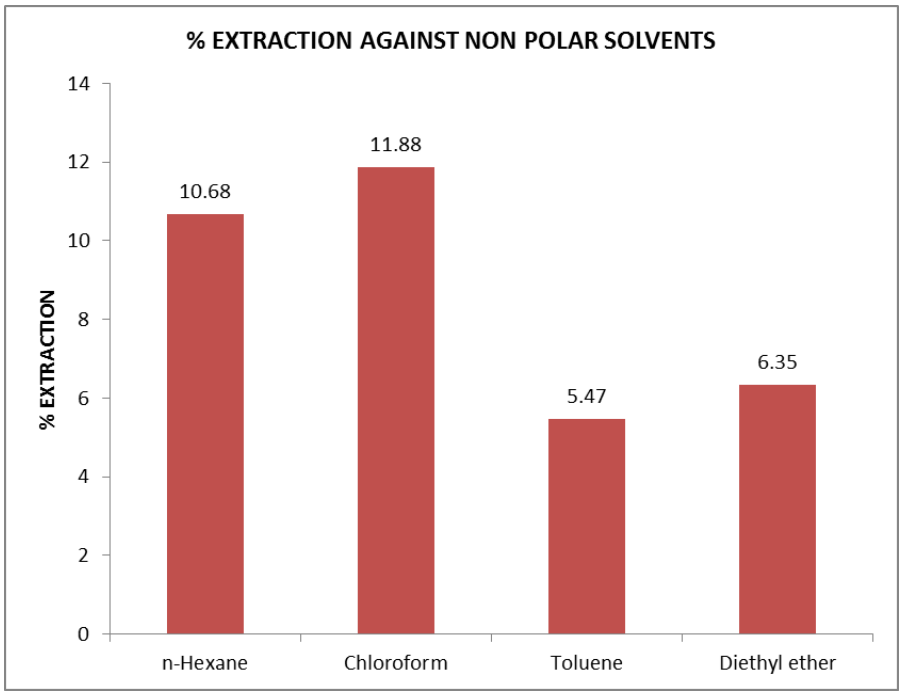

Fig 5: Solvent-Solvent Extraction with Non-polar solvent

Qualitative Phytochemical screening analysis of the T. terrestris extract in n-hexane, chloroform, toluene and diethyl ether was tabulated in table 5.

Table 5: Qualitative data of biomarkers in various solvent mixtures

Obs. No.	Solvent	Alkaloid	Saponin	Flavonoid	Fatty acid
1	n-Hexane	-	++	+	++
2	**Chloroform**	**+**	**++**	**+**	**++**
3	Toluene	-	-	+	++
4	Diethyl ether	-	-	-	+

+ Weakly positive (presence), ++strongly positive (presence), - Negative (absence)

Optimization of numbers of non-polar solvent extraction

From the graph, number of extraction versus percentage extraction is shown Figure 6 and Table 6. It was observed that the percentage extraction remain constant after certain number of extraction.

Table 6: Optimization of Number of Extraction

Obs. No.	No. of Extractions	% Extraction
1	1	10.56
2	2	11.30
3	3	12.34
4	4	12.29
5	5	12.38

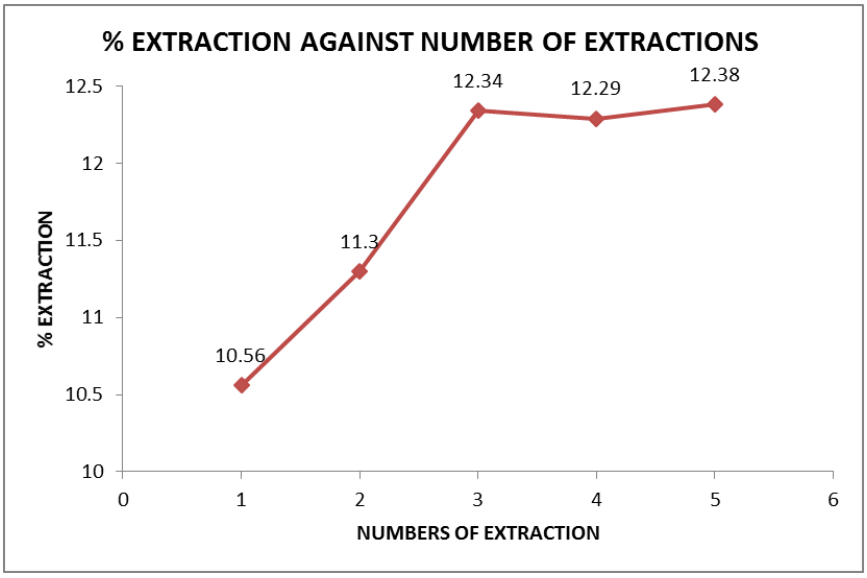

Fig 6: Optimization of number of extraction

Table 7 shows the summary of the optimized conditions for extraction from the fruit powder of *Tribulus terrestris* L.

Table 7: Summary of optimized conditions

Weight of fruit powder	5 gm
Extraction solvent	2 N HCl : Methanol (20:80)
Extraction method	Reflux at 80 °C
Volume of extraction solvent	150 mL
Extraction time	8.0 hrs
Non-polar solvent	chloroform
Number of Extraction	3 times
% Extraction	12.34%

LCMS (Liquid chromatography- mass spectroscopy) analysis

Tribulus terrestris L. fruit sample, extracted with optimized condition was scanned on LCMS system on positive ion mode (Q1MS +VE) in figure 7,shows many $[M + H]^+$ ions out of which 213.0 and 415.2 were further confirmed with fragmentation m/z 213.0 → m/z 198.2, m/z 415.10 → m/z 271 as Harmine and Diosgenin respectively. While on negative mode (Q1MS–VE) in figure 8, shows many $[M - H]^-$ ions out of which 284.9 and 281.0 were further confirmed as Kaempferol and Oleic acid respectively. Simultaneous LCMS-MS (MRM) method also has been developed for the quantification of Harmine, Kaempferol, Oleic acid and Diosgenin.

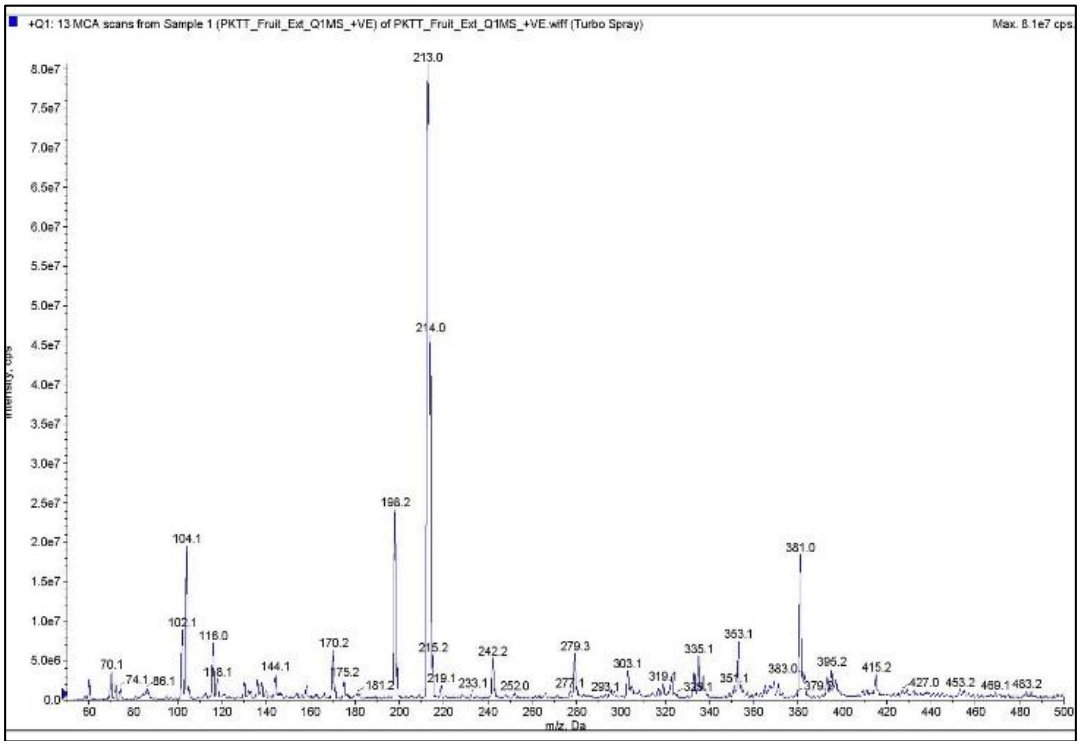

Fig 7: Q1MS +VE mode scan of *Tribulus terrestris* L. fruit sample

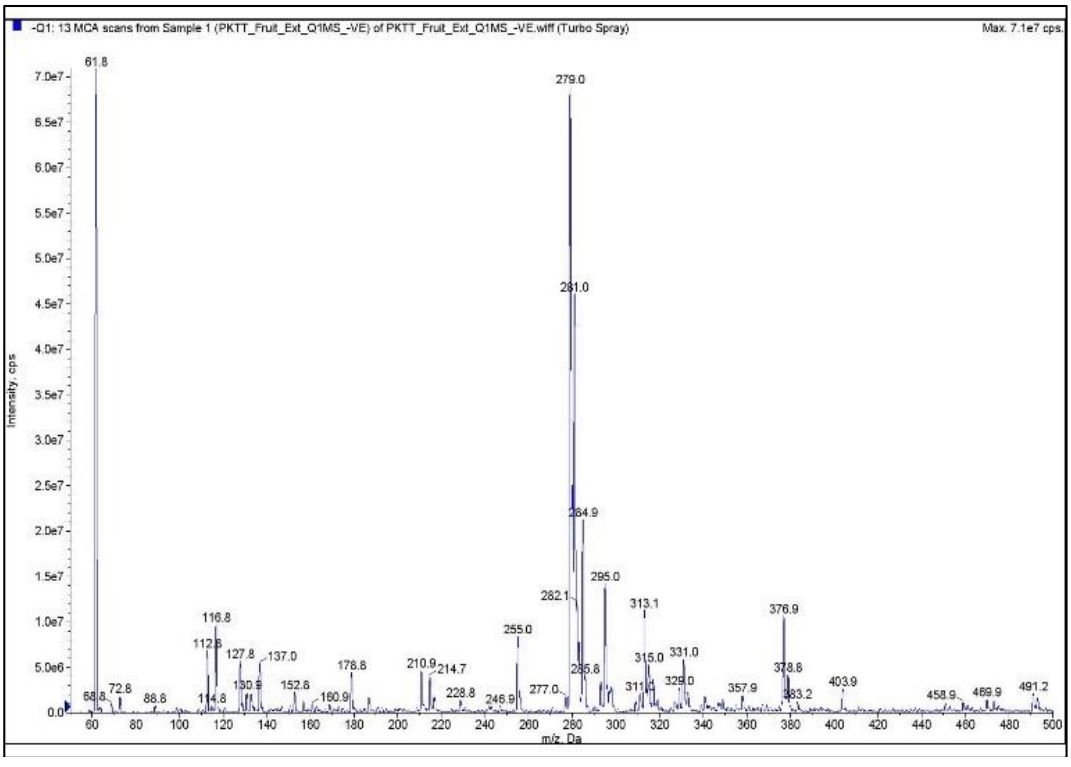

Fig 8: Q1MS -VE mode scan of *Tribulus terrestris* L. fruit sample

Conclusion

Reproducible solvent reflux extraction protocol has been developed for simultaneous identification and enrichment of four bioactive markers Harmine, Kaempferol, Oleic acid and Diosgenin which are known to have anti-diabetic activity. Proposed extraction method can be used as an analytical tool for quality evaluation of plants and formulations containing Harmine, Kaempferol, Oleic acid and Diosgenin as chemical markers. It is an efficient method to screen *Tribulus terrestris* L. fruit samples in order to assess its quality and authenticity.

Conflicts of interest

The authors have declared no conflicts of interest.

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