Appraisal of bioactive compounds isolated by using different solvent system and characterized through LC-MS and HPLC from *Momordica balsamina:* revealed a diverse pharmacological potential

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Abstract: The current study aims to explore the pharmacological potential of Momordica balsamina fruit by characterising its bioactive components. Polarity based extraction was carried out with methanol, ethanol, methanol+ethanol (1:1) and methanol+ethanol (extract pooled) to get concentrated crude extracts of biocativesTo verify the biological activity, crude protein extraction was also carried out. The LC-MS profiling of *M. balsamina* enlists compounds of different classes such as triterpenoids, flavonoids, coumaric acid derivatives, xanthones, quinolines, glycosides, and monoterpenoids. The HPLC results showed that 18 phenolic acids were detected, including 3 unknown phenolic acids (not identified) and three classes of compounds such as cinnamic acid derivatives, benzoic acid derivatives and aldehyde derivatives. By using reported standard protocols, antioxidant, antibacterial, antifungal, anti-inflammatory, antidiabetic and cytotoxic activity was carried out to assess the potential of compounds. The maximum efficacy was obtained from methanol+ethanol(1:1) as compared to other extracts in selected plants. Maximum growth inhibition zones (23.66±2.86) were seen in gram positive bacterial strains in case of ethanol. MTT assay was performed to determine the cytotoxic activity of all crude extracts using HepG7 cell lines. Methanol+Ethanol (1:1) extract showed remarkable cytotoxic effect against HepG7 cell lines, reducing % viability. For in vivo antidiabetic studies specifically targeting diabetic cardiomyopathy, Methanol+Ethanol (1:1) extract was chosen. *Momordica balsamina* showed significant improvement in serum insulin levels of diabetic mice administered regularly with 500mg/kg of plant extracts. The results showed decline in the serum LDH and CK-MB levels as compared to diabetic control groups. Significant decline in Troponin-I level was also observed in our treated groups. Moreover, histopathological analysis of heart and pancreas in diabetic, normal and in treated mice groups indicate the protective effects of the selected plant extracts on cardiac and pancreatic functions. Our study's findings revealed that *M. balsamina* native to Pakistan have great pharmacological significance and an ideal candidate to use as medicine.

Key words: Characterization, Bioactive Compounds, Antioxidant, Antibacterial, Antidiabetic, Anti-Inflammatory, MTT assay

Introduction

Using medicinal plants for healing is as old as mankind. Humans have endured a wide range of diseases, discomforts, and attempts to antagonize them through different means over the course of time (Ferroni, P., et al., 2018). Among the many tactics employed to treat illnesses is the use of medicinal plants to treat a variety of disorders. There are strong evidences from a variety of sources, including historical monuments, written documents plus the original plants-based medicines, that man and his search for therapies in nature have a long history (Lawal, B., et al., 2017, Kruk, J., et al., 2019). Mankind became aware of the use of medicinal plants discovering that the seeds, barks, fruit bodies, as well as other plant parts might be used as remedies (Rasool, A., et al., 2020, Dar, R.A., et al., 2023).

Phytochemicals are a broad class of substances produced by plants that have been shown to be effective in treating a variety of ailments due to their strong antioxidant characteristics (Nwozo, O.S., et al., 2023). Carotenoids, polyphenols, vitamins, and minerals are the four primary groups of natural antioxidants that can be used to group the antioxidants found in fruits and vegetables (Singla, R.K., et al., 2019). Polyphenols and some secondary bioactives, including isoflavones, anthocyanins, resveratrol, pterostilbene, catechins and quercetin have been shown to exhibit strong antioxidant activity (Shahrajabian, M.H. and W. Sun., 2023). They are produced by plants as secondary metabolites. Thus, in the pharmaceutical and medical fields, phytochemicals have the potential to improve health and treat and prevent a variety of illnesses.

It has been estimated that the genus Momordica is quite vast, with roughly 80 species. In traditional medicine, Momordica plants (family: Cucurbitaceae) are commonly used to cure a variety of ailments. *Momordica balsamina* is a monoecious annual herbaceous plant native to subtropical and tropical parts of Asia, Africa, and Australia. They are members of the Cucurbitaceae family (Omokhua-Uyi, A.G. and J. Van Staden, 2020). The leaves of the *Momordica balsamina*, or balsam apple, are used to treat various illnesses in people. It is also consumed as a vegetable in many nations, including Pakistan (Khan, M.N. and L.J.A.E.S. Badshah, 2019). *M. balsamina* leaves have a pale green colour. The fruits of *M. balsamina* take on a spindle-like form and change colour from orange to a vivid scarlet (Flyman, M.V. and A.J. Afolayan, 2007).

Different plant's parts contain a variety of chemicals, including alkaloids, resins, flavonoids, steroids, glycosides, saponins, and terpenes. Many different substances, including terpenoids, alkaloids, flavonoids, mucilages, and reducing chemicals, have been identified in the aerial section of *M. balsamina*. Momordica species are rich in phenolic compounds, epicatechin, gallic acid, catechins, chlorogenic acid, and gentisic acid having chemical formula 2,5-dihydroxyl benzoic acid. The different parts of the plant contain a multitude of chemicals, including as alkaloids, resins, flavonoids, steroids, glycosides, cardiac glycosides, saponins, and terpenes. The fruit of *M. balsamina* is notable for having a large amount of carbohydrates, steroid rings, and saponins (Ramalhete, C., et al., 2022; Flyman, M.V. and A.J. Afolayan, 2007).

The potential medical benefits of these bioactive components have sparked an increased interest in their identification and extraction. Thanks to advancements in technology, the processes of phytochemical screening, phytochemical extraction, and analytical characterization of bioactive compounds from plants have become incredibly straightforward. Therefore, it would be beneficial to assess the antioxidant principles and other pharmacological activities of M. balsamina, which is readily available in the area, in order to investigate their prospective use as natural antioxidants and preservatives in the food and pharmaceutical industries. In light of this, the present investigation examined the M. balsamina's total phenols, total flavonoids, and antioxidant qualities in addition to its composition of specific phenolic acids.

1. Materials and methods:

1.1 Sample collection and preparation

In this investigation, *Momordica balsamina*. was obtained from the Sargodha district of Pakistan. Dr. Amin Ullha Shah, a botanist from the University of Sargodha's Department of Botany, verified the authenticity of the plant that had been obtained. After that, fruit part of the plant was cleaned and allowed to air dry. The samples were subsequently processed using an electronic grinder to a coarse powder.

1.2 Crude extraction with multiple solvent systems

The chosen samples were combined with different solvents, such as absolute ethanol, absolute methanol, and 1:1 ethanol and methanol, to make the crude bioactive component extracts. The solvent to sample ratio was maintained at 1/10 (W/V). Samples were soaked in the

respective solvents for two days, and then the solution was shaken for twenty-four hours using orbital shaker. All of the aforementioned sample solutions are filtered using muslin cloth (folded three times) after being shaken for a full day. After the first filtration, the residue was combined with more solvent and shaken again on an orbital shaker. For all the solvents, the same procedure was repeated three times. All of these solvents were evaporated using a hot plate, and the resulting extracts were refrigerated at -20°C to be used in subsequent analytical testing.

1.2.1 Extracts pooling

Momordica balsamina absolute ethanol extracts and absolute methanol extracts were combined in an equal ratio and allowed to soak in methanol for ten days. For the investigation, pooled extracts were utilized.

1.2.2 Total protein extraction

For Total protein extraction, protocol from Lledías, F., et al., 2017 was used.

1.3 Extraction of bound phenolic acids

Extraction of bound phenolic acids was done by base hydrolysis assisted by ultrasonication. 10 ml of base solution mixture (0.372 g EDTA and 1 g of ascorbic acid in 2 N NaOH) was used to hydrolyze the sample (ground powder) followed by nitrogen purging before capping. 50 mL propylene tubes were used for capping, then they were vortexed and left for sonication at 56°C for 30 min (Memon A.A. et al, 2013). Later, pH of cooled samples was adjusted with 6 N HCl and phenolic acids were extracted out by using 5 × 2 mL of ethyl acetate. Further, for 20 sec, mixtures were vortexed and centrifuged at 5000 rpm on counter centrifuge (H-200NR Kokusan, Japan) for 10 min to get the aqueous and organic layers separate. Upper organic layer containing the bound phenolic acid was transferred into separate tube following the drying of combined organic layers in a nitrogen stream. Obtained dried residues further dissolved in 2mL of 80% aqueous methanol. Again, mixtures were vortexed and filtered with PVDF filters (0.45 μm) before subjecting to HPLC analysis.

1.4 Liquid Chromatography- Mass Spectrometer (LC-MS) analysis of extracts

Using the previously published approach by Kim et al. (2018), the phytochemical profiling of many extracts of *Momordica balsamina* were carried out by LC-MS. Thermo Scientific's Finnigan

TSQ Quantum Ultra EMR was utilized for this purpose. An extensive library of chemicals was found in the LC-MS chromatogram, and this was verified by means of the extensively utilized Human Metabolom Database (HMDB).

1.5 Separation of bound phenolic acids by HPLC-DAD

Separation of phenolic acids from the obtained extracts was performed by using a SCM 1000 Spectra system (ThermoFinnigan, California, USA) liquid chromatograph coupled with a diode array detector (DAD) system. Hypersil Gold C-18 (250 mm × 4.6 mm, 5 μm) reverse-phase column (Thermo Corporation, USA) was used for separation, preceded by a guard column of the same packing material (Memon A.A. et al., 2013). The gradient mobile phase consists of solvent A (formic acid 0.1% in water) and solvent B (water). The flow rate was set to be 1 mL.min–1. The linear gradient was 5%(A) to 30% (A) for 25 min, followed by 30% (A) for 45 min, from 45.1 min 100% (A) up to 52.0 min, then elution was brought back to 5%(A) up to 55 min for column equilibration. Using DAD, UV analysis was performed at 270, 310 and 325 nm. To interpret data, Chromquest Version 4.2 software was used. Retention time (tR) and UV spectra of the standards helped in confirming the structures of all the phenolic acids. Standard solutions ranged in the various concentrations, 1-50 $\mu g.mL^{-1}$, introduced into the spectra system for the construction of each standard curve. The concentrations of the compounds were calculated from peak area in accordance with their respective standard curves.

1.6 Estimation of antioxidants

1.6.1 Total Phenolic Contents (TPC)

With minor adjustments, the Folin-Ciocalteu test was used to measure the total phenolic content, following the methodology described by certain authors (Waterhouse 2002). Three different types of solutions were made for the experiment: a 10% folin-Ciocalteu solution in water, a 7.5% sodium carbonate solution in water, and a 2 mg/ml extract (2 mg extract diluted in 1 ml methanol). Triplicates of the protocol were executed. Each sample's 300μl (0.3 ml) extract solution was collected and placed in a different test tube. Each test tube was filled with 2 ml of a 10% Folin-Ciocalteu solution, and it was then incubated for 7 minutes. Samples were placed in the dark for 30 minutes after adding sodium carbonate (7.5%). The reference standard that was employed was Gallic acid. Absorbance was taken at 765 nm using a blank. The results were represented as

milligram Gallic acid equivalents per gram of extract after the total phenolic content was estimated using the Gallic acid standard curve (Kalpna et al. 2011).

1.6.2 Total Flavonoids Contents (TFC)

With some adjustments, the Zhishen et al. (1999) technique was used to assess the TFC. 1 ml of methanol (2 mg/ml) was used to dissolve 2 mg of extract, and the resultant mixture was then further diluted in 2 ml of distilled water. After adding 0.15 ml of 5% NaNO2 solution, the mixture was incubated for five minutes. Following the addition of 0.15 ml of a 10% AlCl3 solution, the samples were incubated for a duration of 6 minutes. Then, the samples were incubated for five minutes after one milliliter of a 1M NaOH solution was added. Against a blank, absorbance was measured at 510 nm. Readings were expressed as milligrams of catechin equivalents per gram of extract, with catechin serving as the reference standard.

1.6.3 1, 1-Diphenyl-2-picrylhydrazyl Hydrate (DPPH) Radical Scavenging Assay

Using a slightly modified version of the process outlined by Aldughaylibi et al., 2022, the antioxidant capacity of the plant extracts was assessed using the DPPH Radical Scavenging Assay. 12 mg weighed sample was dissolved in 1 ml of DMSO. The concentrations were then serially diluted (6 mg, 3 mg, 1.5 mg and 0.75 mg). 100µl of each concentration was added to a distinct well of a 96-well plate (with the sample ID carefully recorded). Next, 100µl of DPPH solution (0.1 mM in methanol) was added. The positive control in this experiment was Butylated Hydroxytoluene, while the negative control was DPPH solution (which does not contain any antioxidant). After that, the samples were incubated in the dark for thirty minutes (all preparations were carried out in low light because DPPH is very light sensitive). At 517 nm, absorbance was measured. The following formula was used to determine the percentage of scavenging/inhibition:

$$\textbf{Scavenging Inhibition (\%)} = \frac{(\textbf{Absorbance}_{\textbf{control}} - \textbf{Absorbance}_{\textbf{sample}})}{\textbf{Absorbance}_{\textbf{control}}} \textbf{ X } \textbf{100}$$

Whereby

$Absorbance_{sample}$ = absorbance of extract

$Absorbance_{control}$ = absorbance of negative control

1.7 Antimicrobial activity

The Disc Diffusion Method was used to evaluate the plant's potential to prevent the growth of a particular type of bacterium and fungus. Two bacterial strains, namely *Escherichia coli* and *Streptococcus spp*., were used for this study. The fungal strains used in study were namely *Aspergillus flavus* and *Fusariu oxysporum*. Antifungal and antibacterial drug fluconazole and erythromycin used as a positive reference. Petri plates were filled with autoclaved LB agar. On each plate, 20 µl of bacterial and fungal culture (cultivated in LB broth) was poured. A total of three wicks paper discs were placed onto each plate, with each disc holding 50 µl of a 1mg/ml extract (the extract was placed on two discs, and the erythromycin/ fluconazole disc was placed on the third). After each plate was labeled, it was securely wrapped in parafilm and incubated for twenty-four hours at 37˚C. The discs developed inhibition zones, which were subsequently measured in millimeters using a scale (Sarpeleh, A., K. Sharifi, and A. Sonbolkar., 2019).

1.8 Cytotoxic activity (MTT Assay)

To evaluate the cytotoxicity of *M. balsamina* plant extracts, standard MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colorimetric assay was used. In this research, the HepG7 cell lines were used. After dissolving 100 mg of each extract in 1 milliliter of DMSO, the samples were serially diluted twice (12 mg, 6 mg, and 3 mg). The cell lines were cultivated in MEME (Minimum Essential Medium Eagle), supplemented with 5% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. The cultures were allowed to grow at 37 °C with 5% CO2 supplied. A cell culture with a concentration of 6 x 10^4 cells/mL was prepared after cell harvesting. A 96-well plate was filled with 100 µl of the solution (which contained the cells and medium) and left to incubate overnight. After removing the medium, 100 µl of extract solution or different concentrations was added. It was then incubated at 37^{o}C for 24 hours in 5% CO_2 concentration. For standard, filtered DMSO was used. Following a 24-hour incubation period, 200 µl of MTT (500 µg/ml) was added into each well. After reducing MTT to formazan with 100 µl of DMSO, absorbance was measured at 570 nm.

1.9 Anti-inflammatory activity

With slight adjustments, the Williams et al. (2008) procedure was used to assess the in vitro anti-inflammatory potential. Extracts from *M. balsamina* were examined using the protein denaturation inhibition technique. After dissolving 12 mg of each extract in 1 ml of DMSO, the extracts were

serially diluted (6 mg, 3 mg, 1.5 mg, and 0.75 mg). A 96-well plate was filled with 50µl of each plant extract, and then 100µl of BSA solution (0.2% w/v BSA dissolved in water) was added. After 20 minutes of incubation at 37 °C, the solutions were placed on a water bath set at 60 °C for a duration of 15 minutes. BSA served as the negative control and diclofenac as the positive control. The absorbance was measured at 660 nm using a blank. This procedure was performed in triplicates. The formula for %age inhibition was as follows:

$$\textbf{Inhibition (\%)} = \frac{(\textbf{Absorbance}_{\textbf{control}} - \textbf{Absorbance}_{\textbf{sample}})}{\textbf{Absorbance}_{\textbf{control}}} \textbf{ X } \textbf{100}$$

Whereby

$Absorbance_{sample}$ = absorbance of extract
$Absorbance_{control}$ = absorbance of negative control

1.10 Anti-diabetic assay

To determine the anti-diabetic potential of plant extracts from *M. balsamina*, a thorough in-vitro assay was carried out (Vyas, M. 2017). The concentrations of each sample, which ranged from 12 mg, 6 mg, 3 mg, 1.5 mg, 0.75 mg per milliliter, were produced in DMSO. 390 µl of each produced extract solution was dissolved with 10 µl of the α-amylase solution, and incubated at 37°C for 10 minutes. After adding 100 µl of 1% starch solution, the samples were incubated for 60 minutes. After the incubation period, each sample was mixed with 0.1 ml of 1% iodine solution. As a positive control, metformin was employed. At 565 nm, the absorbance was measured. The percentage. The following formula was used to calculate percentage inhibition:

$$\textbf{Inhibition (\%)} = \frac{(\textbf{Absorbance}_{\textbf{control}} - \textbf{Absorbance}_{\textbf{sample}})}{\textbf{Absorbance}_{\textbf{control}}} \textbf{ X } \textbf{100}$$

Whereby

Absorbance $_{sample}$ = absorbance of extract
Absorbance $_{control}$ = absorbance of negative control

1.11 In-Vivo Study

1.11.1 Experimental animals

Animal study was conducted after obtaining ethical review board approval. Thirty albino mice, weighing 25–35 grams and never having been used in a study before, were chosen for the intended investigation. Throughout the whole study period, they were fed a regular diet.

1.11.2 Experimental groups

Four groups were created with five rats in each group shown in table 1 (Airaodion, A.I., et al., 2019).

Table 1. Experimental groups

Groups	Treatment
1	Control group (Normal)
2	Negative control: Untreated diabetic mice
3	Positive control: Glucophage treated diabetic mice (5mg/kg)
4	Diabetic mice treated with ethanol+methanol(1:1) Extract (500mg/kg)

1.11.3 Induction of diabetes

Alloxan Monohydrate was utilized to induce diabetes because it destroys the pancreatic β cells, which results in diabetes (Macdonald Ighodaro et al. 2017). After a 16-hour fast, mice received an intraperitoneal injection of 150 mg/kg of Alloxan monohydrate. After the injection, blood glucose

levels were checked 72 hours later, and models with blood glucose levels greater than 180 mg/dl were chosen for further investigation (Pareek and Suthar 2010).

1.11.4 Preparation of plant extract dose

The dosage was determined using methanol+ethanol(1:1) extract from *Momordica balsamina*, as in-vitro anti-diabetic and antioxidant experiment indicated that it had highest potential. The dosage was made at 500 mg/kg or body weight in 0.9% Normal Saline.

1.11.5 Preparation of Glucophage dose

The dosage of Glucophage was prepared in about 0.9% Normal Saline at a concentration of 5 mg/kg of mice body weight.

1.11.6 Blood glucose monitoring

Mice's blood glucose levels were routinely checked using a Medisign MM800 Auto glucometer on days 1, 7, 14, 21, and 28.

1.11.7 Slaughtering and biochemical tests

Blood was taken from the heart's aorta after slaughtering, following the animals' 28 days of observation and care (Lotfi and Safamanesh 2022). Blood was drawn from heart into an EDTA vial, and it was centrifuged at 2500 rpm to separate the serum.

1.11.8 Serum Insulin

Serum insulin levels play a critical role in the diagnosis and treatment of a number of disorders pertaining to insulin function and glucose metabolism. Serum samples were prepared by centrifugation for 15 minutes at 2500 rpm at room temperature.

1.11.9 Cardiac Biomarkers

1.11.9.1 Serum LDH

The enzyme lactate dehydrogenase (LDH) is measured in serum levels. This test keeps track of and identifies diseases involving tissue damage in the heart, liver, and muscles, among other organs. Increased LDH levels are indicative of cardiac myopathy, or other heart-related conditions. Samples of serum were prepared by centrifugation at 2500 rpm for 15 minutes at room temperature.

1.11.9.2 CK-MB

Creatine Kinase-MB, or CK-MB, is a cardiac biomarker that is used to identify cardiac muscle and function impairment. One characteristic that many cardiac myopathies have is increased CK-MB levels. Damage to the heart muscle is indicated by elevated CK-MB levels. Serum samples were prepared for this by centrifugation for 15 minutes at 2500 rpm at room temperature.

1.11.9.3 Troponin I

Heart muscle-specific cardiac marker is troponin I. Increased troponin I levels suggest myocardial infarction and validate the diagnosis and severity of the cardiac attack. Serum samples were prepared for this by centrifugation for 15 minutes at 2500 rpm at room temperature.

1.11.10 Histological analysis

The study concluded with the mice being killed (mercy killed) and their peritoneum being meticulously dissected. After being removed, the pancreas and heart were preserved in a 10% formalin solution (Ahmed, M.F., et al., 2010).

1.11.10.1 Slide Preparation

For the preparation of slides, primary step employed are shown in figure 1.

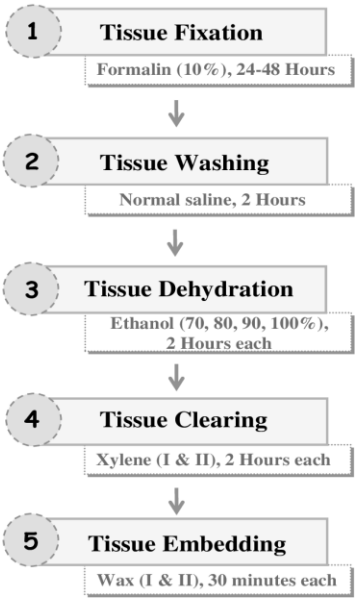


Figure 1. Stages of histopathology slides preparation

1.11.10.2 Tissue Sectioning

A microtome was used to cut the paraffin-embedded heart and pancreas tissues into thin slices that ranged from 3 to 5 μm. The slices were spread out over a 40°C water bath, captured on slides, and then dried for 30 minutes at 60°C.

1.11.10.3 Tissue Staining

Slides were stained with hematoxylin and eosin stains (Ankle, M.R. et al., 2011). Steps involved are summarized in figure 2.

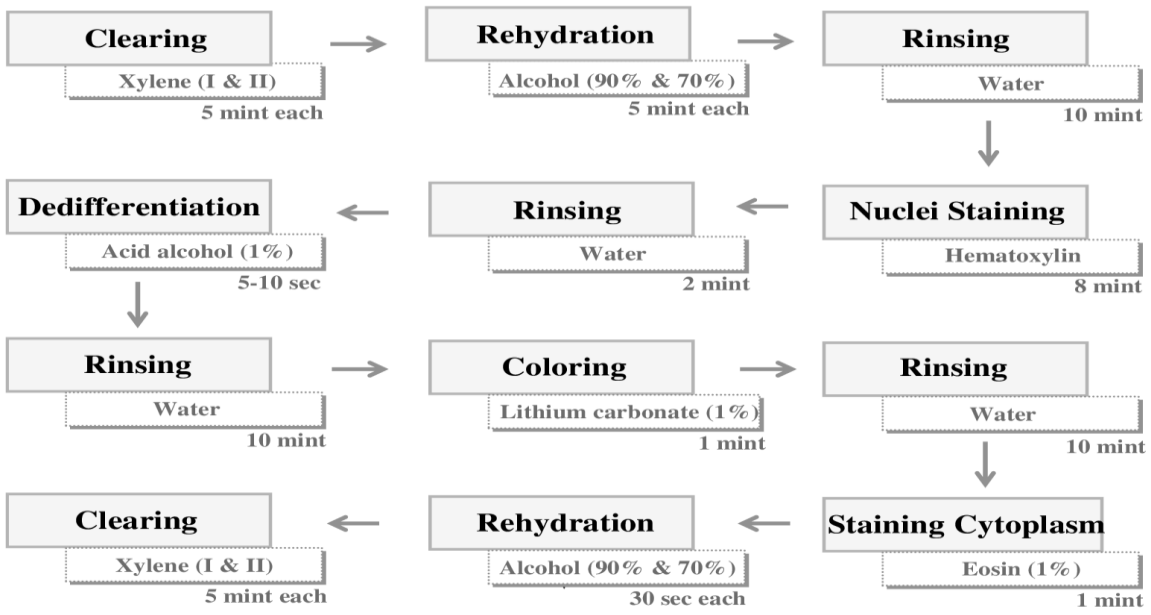


Figure 2. Haematoxylin and Eosin staining protocol

1.11.10.4 Mounting

Careful placement of .Dibutyl phthalate polystyrene xylene(DPX) and a coverslip on the slide allowed the slides to be viewed via a metallurgical microscope equipped with a camera.

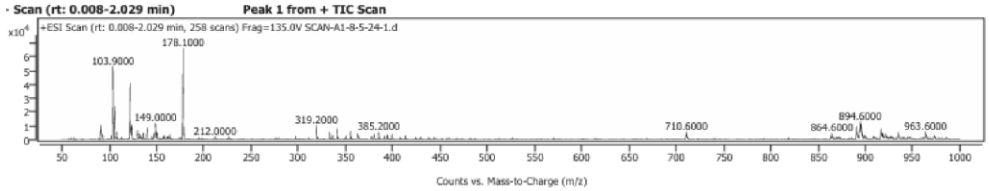
1.12 Statistical analysis

All the results are expressed as mean ± SD, further analyzed using ANOVA statistically. Statistical significance was determined by $p < 0.05$.

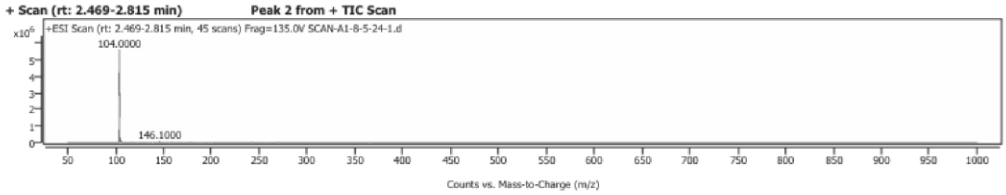
2. Results

Table 2. LC-MS profiling of ethanol extracts from *Momordica balsamina*

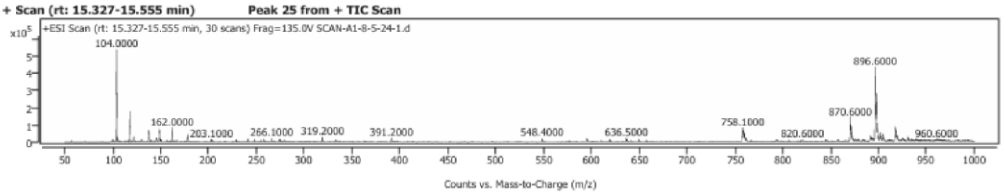
Sr. #	Class	Name	Formula	Mol. weight	Monoisotopic mass	Retention time (min)
1	Organo oxygen compounds	Gluconolactone	$C_6H_{10}O_6$	178.14	178.047738052	0.063
2	Carboxylic acids and derivatives	Malonic acid	$C_3H_4O_4$	104.0615	104.010958616	2.541
3	Carboxylic acids and derivatives s	N-methylanthraniloyl-CoA	$C_{29}H_{39}N_8O_{17}P_3S$	896.65	896.138868315	15.445
4	Flavonoids	Tricin 7-neohesperidoside	$C_{29}H_{34}O_{16}$	638.5707	638.18468504	18.016
5	Flavonoids	Kaempferide 7-glucoside	$C_{22}H_{22}O_{11}$	462.4035	462.116211546	17.623
6	Prenol lipids	Oleuropein	$C_{25}H_{32}O_{13}$	540.518	540.18429109	18.835



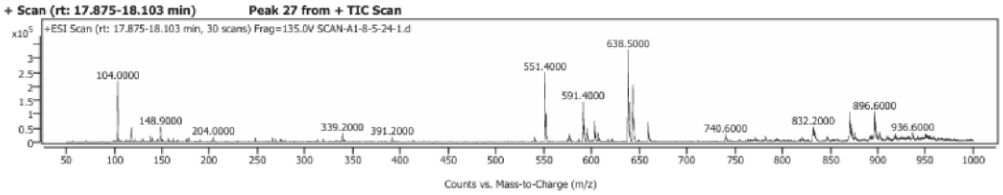
a) Gluconolactone



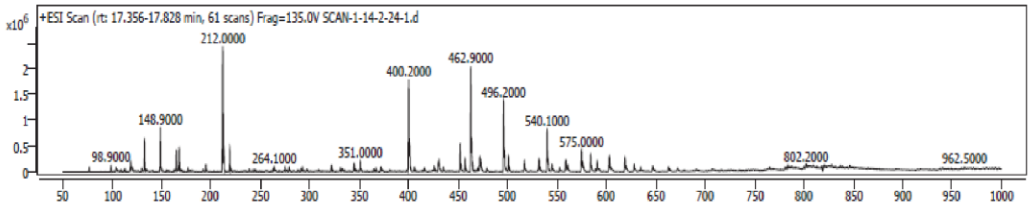
b) Malonic acid



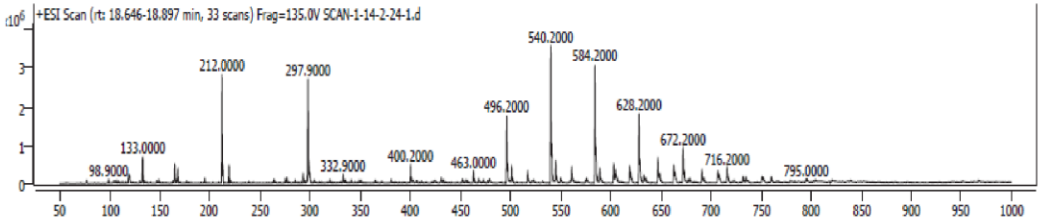
c) N-methylanthraniloyl-CoA



d) Tricin 7-neohesperidoside



e) Kaempferide 7-glucoside



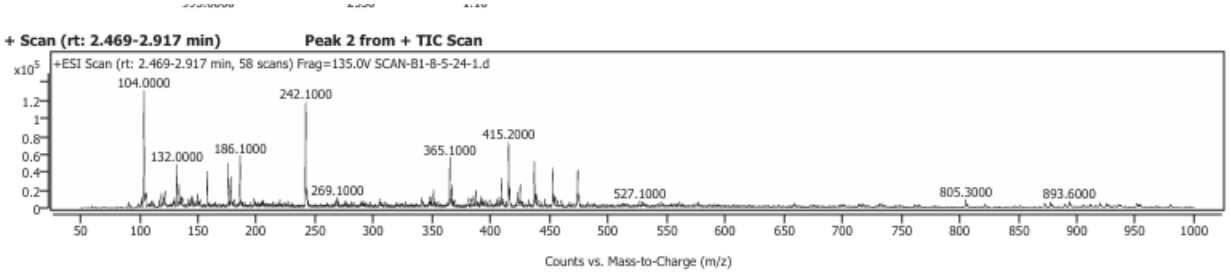
f) Oleuropein

Figure 3: (a-f) LC-MS chromatogram of ethanol extracts from *Momordica balsamina*

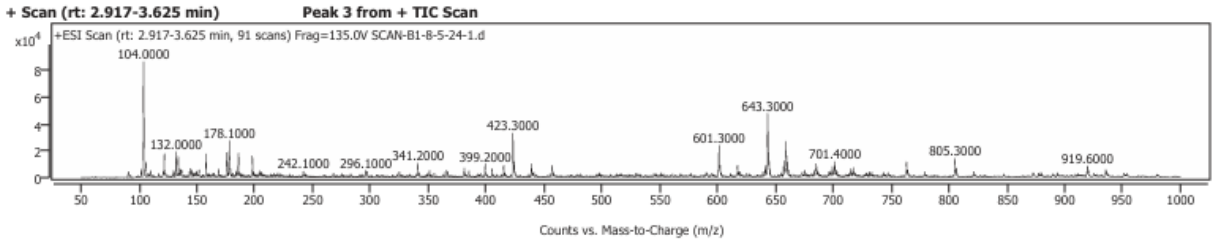
Table 2: LC-MS profiling of Methanol extracts from *Momordica balsamina*

Sr. No	Class	Name	Formula	Mol. weight	Monoisotopic mass	Retention time
1	Organic phosphoric acids and derivatives	2-hydroxy-5-(methylthio)-3-oxopent-1-enyl phosphate	$C_6H_{11}O_6PS$	242.1800	242.0014	2.540
2	Carboxylic acids and derivatives	**Malonic acid**	$C_3H_4O_{4ZZ}$	104.0615	104.010958616	2.980
3	Organic phosphoric acids and derivatives	**Phosphoglycolic acid**	$C_2H_5O_6P$	156.0313	155.9824	10.687
4	Xanthones	**Epicatechin 3-O-gallate-(4beta->8)-epigallocatechin 3-O-gallate**	$C_{44}H_{34}O_{21}$	898.7282	898.15925815	15.665
5	Coumaric acids	**3,4,5-Trimethoxycinnamic acid**	$C_{12}H_{14}O_5$	239.08	238.08	12.488

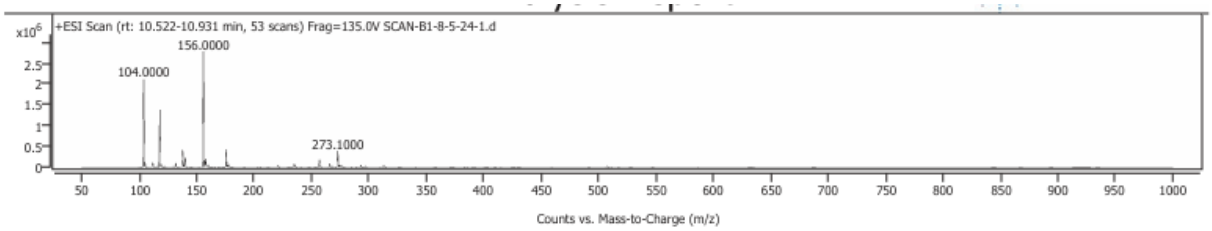
6	Tetraterpenoids	Neoxanthin	$C_{40}H_{56}O_4$	600.8702	600.41786028	24.473



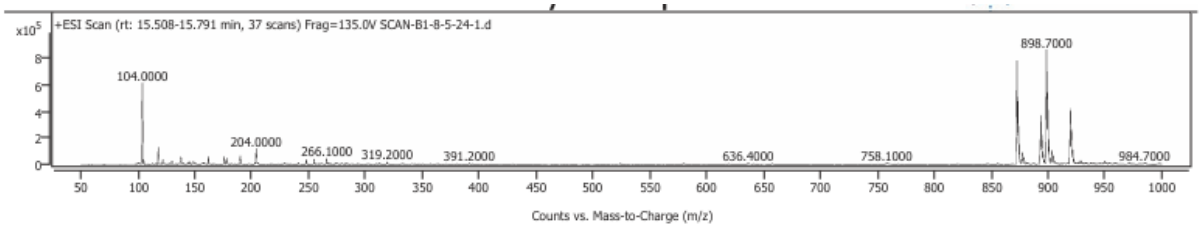
a) 2-hydroxy-5-(methylthio)-3-oxopent-1-enyl phosphate



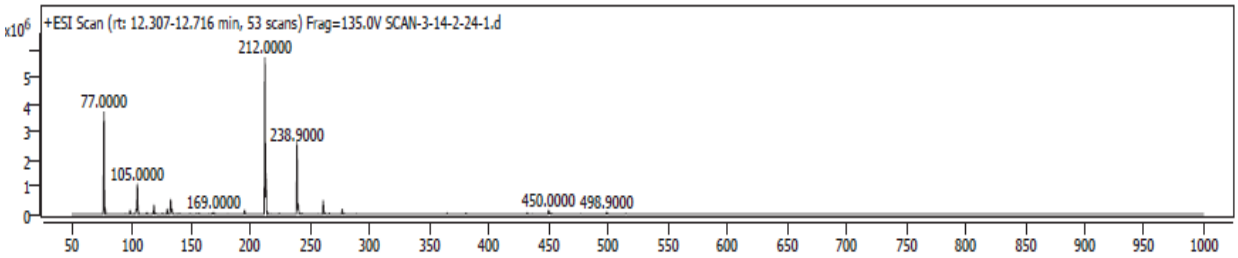
b) Malonic acid



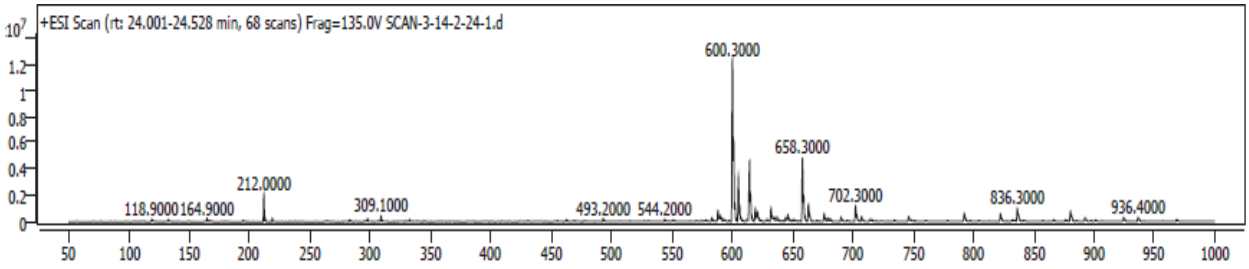
c) Phosphoglycolic acid



d) Epicatechin 3-O-gallate-(4beta->8)-epigallocatechin 3-O-gallate



e) 3,4,5-Trimethoxycinnamic acid



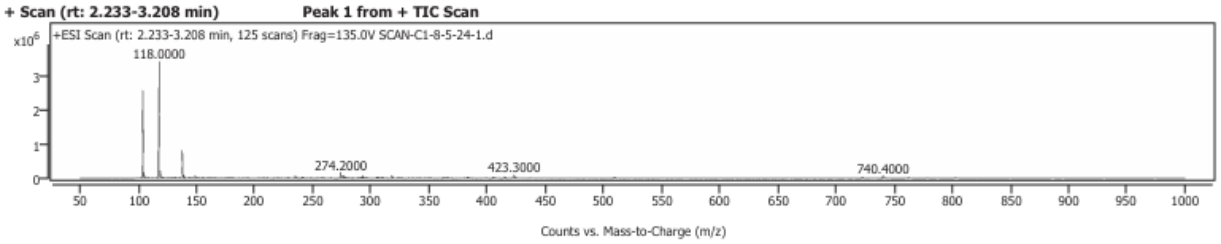
f) Neoxanthin

Figure 4. (a-f) LC-MS chromatogram of methanol extracts from *Momordica balsamina*

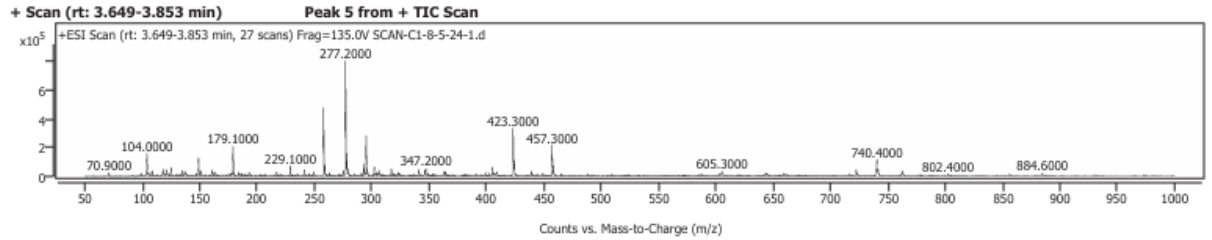
Table 3: LC-MS profiling of ethanol+methanol(1:1) extract from *Momordica balsamina*

Sr. No	Class	Name	Formula	Mol. Weight	Monoisotopic mass	Retention time
1	Dicarboxylic acids and derivatives	Succinic acid	$C_4H_6O_4$	118.088	118.02660868	2.752
2	pyrrolo[2,3-d]pyrimidines	Queuine	$C_{12}H_{15}N_5O_3$	277.2792	277.117489371	3.720

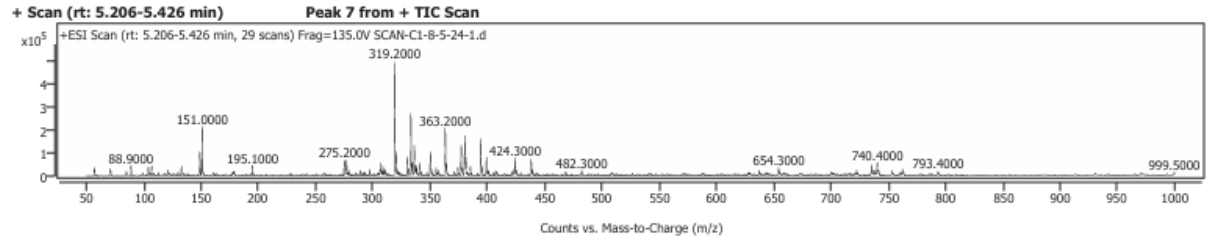
3	Phosphate esters	N-Gluconyl ethanolamine phosphate	$C_8H_{18}NO_{10}P$	319.203	319.066832313	5.355
4	phenolic glycosides	3-(4-Hydroxy-3-methoxyphenyl)-1,2-propanediol 2-O-(galloyl-glucoside)	$C_{23}H_{28}O_{13}$	512.4606	512.152990982	13.172
5	Quinolines and derivatives	Acrimarine I	$C_{34}H_{31}NO_7$	565.6124	565.210052351	14.604
6	2-enoyl coas	Glutaconyl-coa	$C_{26}H_{40}N_7O_{19}P_3S$	879.62	879.131254264	17.812
7	coumaric acids and derivatives	4-Demethylsimmondsin 2'-(E)-ferulate	$C_{25}H_{31}NO_{12}$	537.5131	537.184625461	19.424
8	cytidine diphosphate diacylglycerol	Cdp-dg(16:0/18:1(11z))	$C_{46}H_{83}N_3O_{15}P_2$	980.1098	979.529942025	24.048



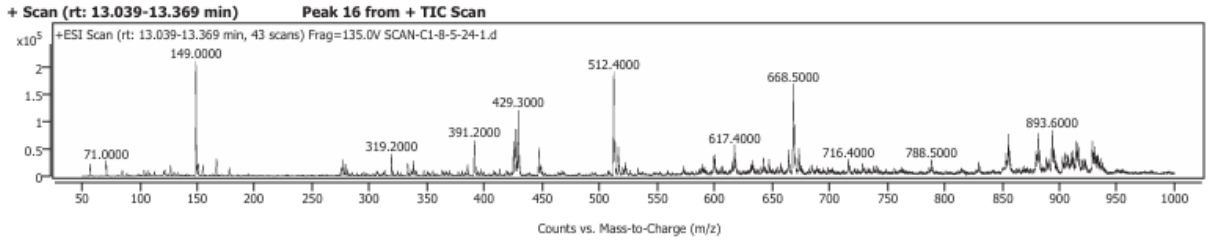
a) Succinic acid



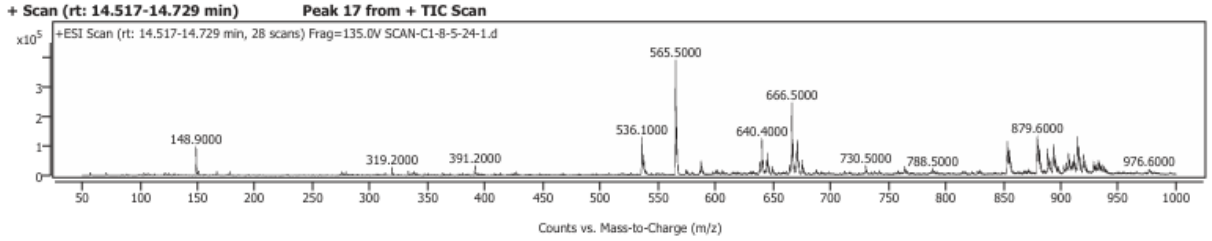
b) Queuine



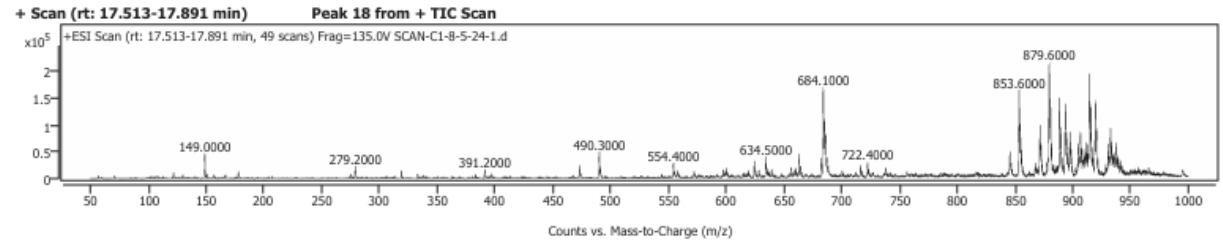
c) N-Gluconyl ethanolamine phosphate



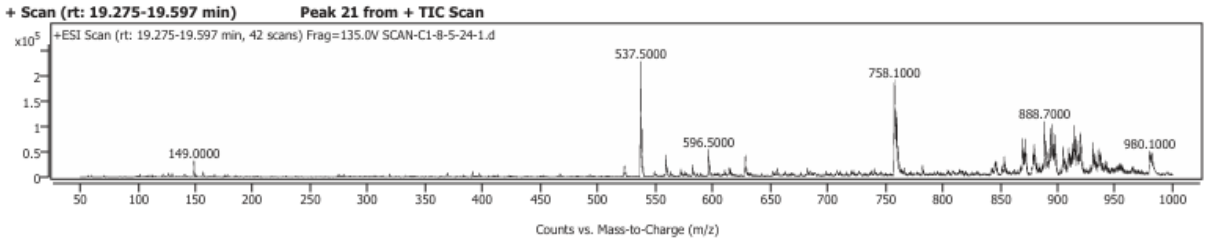
d) 3-(4-Hydroxy-3-methoxyphenyl)-1,2-propanediol 2-O-(galloyl-glucoside)



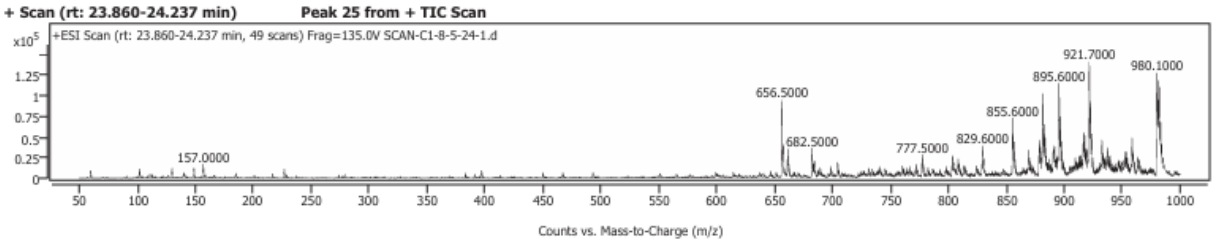
e) Acrimarine I



f) Glutaconyl-CoA



g) 4-Demethylsimmondsin 2'-(E)-ferulate



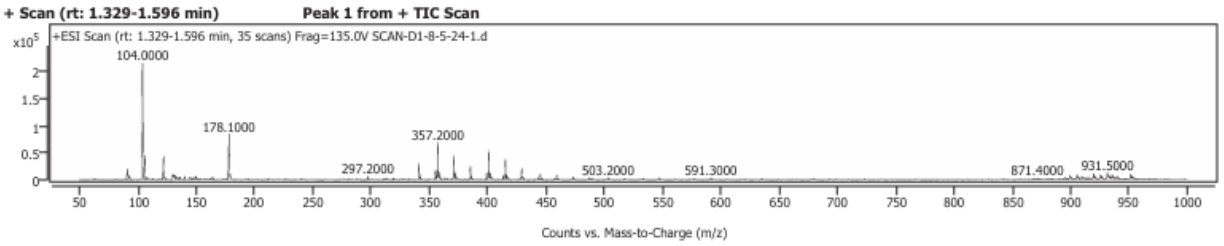
h) CDP-DG(16:0/18:1(11Z))

Figure 5: (A-h) LC-MS chromatogram of methanol+ethanol(1:1) extracts from *Momordica balsamina*

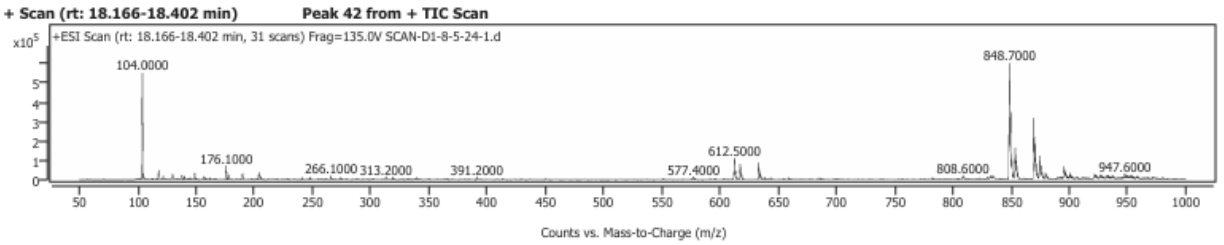
Table 4: LC-MS profiling of ethanol+methanol (pooled) extract from *Momordica balsamina*

Sr. No	Class	Name	Formula	Mol. weight	Monoisotopic mass	Retention time
1	Carboxylic acids and derivatives	Malonic acid	$C_3H_4O_4$	104.0615	104.010958616	1.360
2	Glycerophospholipids	PS(18:0/22:0)	$C_{46}H_{90}NO_{10}P$	848.197	847.6302351	18.292
3	Flavonoids	r-Viniferin	$C_{56}H_{42}O_{12}$	906.9255	906.267626808	19.983

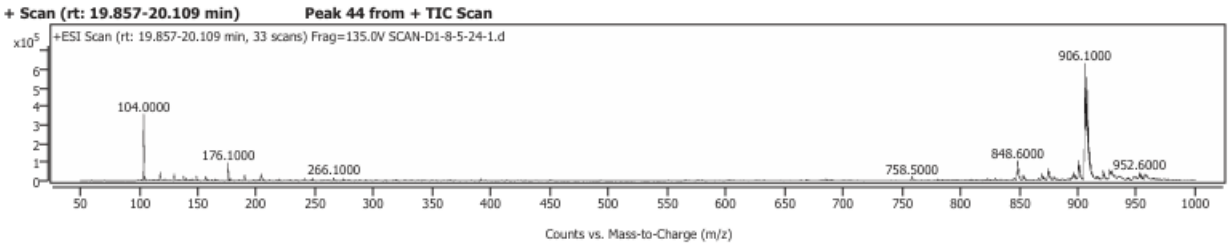
4	Flavonoids	Pelargonidin 3-rhamnoside 5-glucoside	$C_{27}H_{31}O_{14}$	579.5266	579.1713807	21.603
5	Organo oxygen compounds	3'-Glucosyl-2',4',6'-trihydroxyacetophenone	$C_{14}H_{18}O_9$	330.2873	330.095082174	11.348



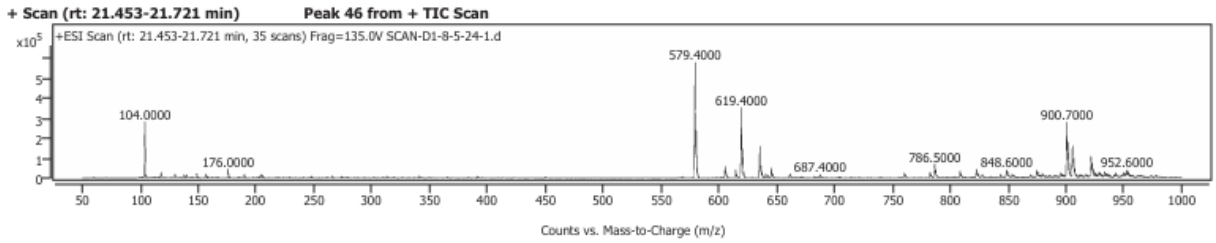
a) Malonic acid



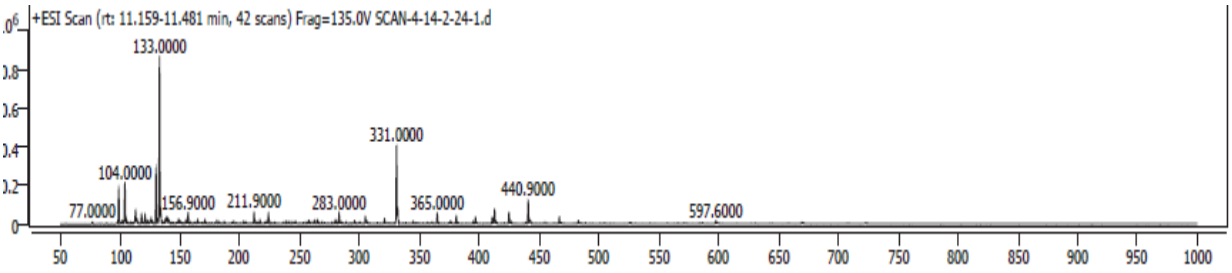
b) PS(18:0/22:0)



c) r-Viniferin



d) Pelargonidin 3-rhamnoside 5-glucoside



e) 3'-Glucosyl-2',4',6'-trihydroxyacetophenon

Figure 6: (a-e) LC-MS chromatogram of methanol+ethanol (pooled) extracts from *Momordica balsamina*

Table 5: TPC and TFC of solvent extracts and protein extract from *Momordica balsamina*

Extracts	**Mean ± S.D TPC as Gallic acid (mg/g)**	**Mean ± S.D TFC as Catechin (mg/g)**
Methanol	22.39± 0.97	43.41±4.60
Ethanol	34.86±0.85	41.88±0.30
Methanol+ethanol (1:1)	40.31±0.59	52.92±0.31
Ethanol+methanol (pooled)	23.78±1.02	35.56±0.47
Crude protein	7.42±0.88	19.28±0.73

Values are means ± SD (n=3) of three separate experiments. All values were statistically significant $p < 0.05$.

Table 7: Antifungal and antibacterial activity of various extracts obtained from *M. balsamina*

Extracts	*E.coli*	*Streptococcus Spp*		*Aspergillus flavus*	*Fusarium oxysporum*
Methanol	19.66±0.94	16.66±1.69		15.43±0.32	12.10±0.35
Ethanol	23.66±2.86	21.66±3.39		9.98±0.19	11.3±0.10
Methanol+Ethanol (1:1)	22.66±3.09	19.33±1.69		18.10±0.10	19.30±0.50
Ethanol+methanol (pooled)	17±2.44	18.66±1.24		17±0.31	16.5±0.21
Crude protein	12±2.16	11.33±2.86		3.23±0.45	2.89±0.34
Erythromycin	27.11±1.09	25.45±1.23	**Fluconazole**	37±0.05	37±0.05

Values are means ± SD (n=3) of three separate experiments. There were significant ($p< 0.05$) differences of means among tested extracts towards disease causing bacteria and fungal strain.

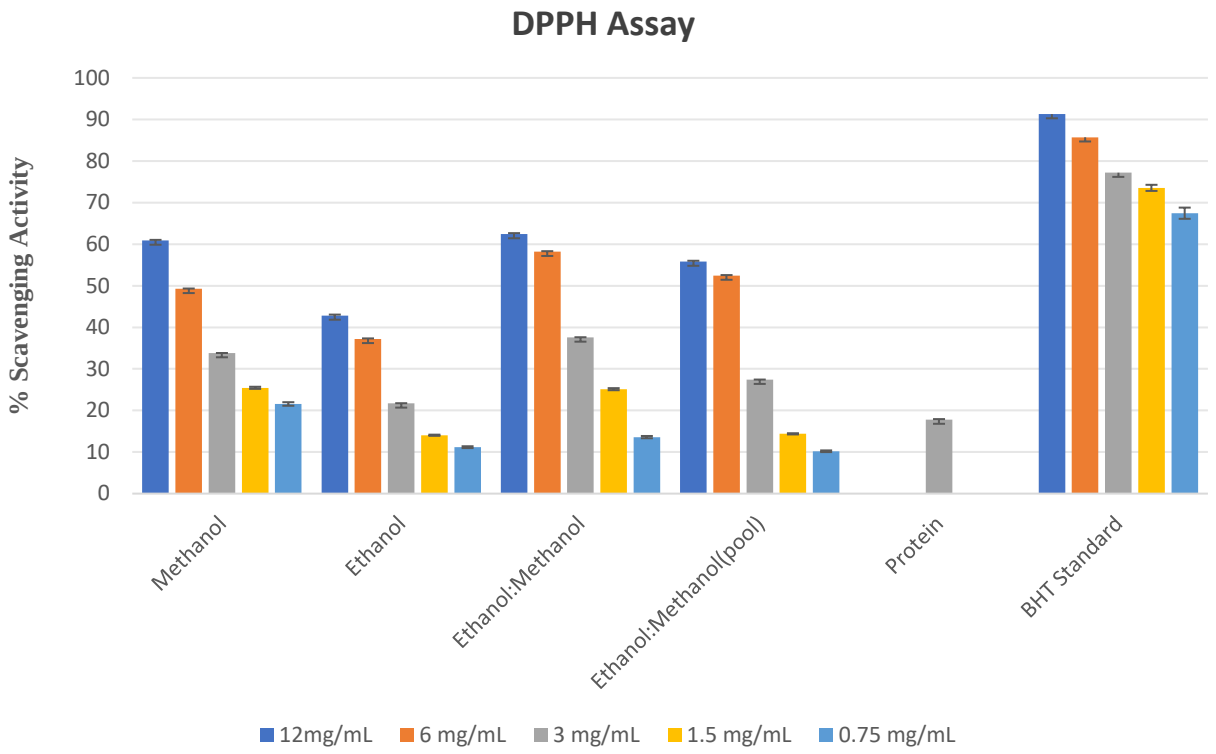


Figure 7: DPPH radical scavenging activity of solvent and protein extracts from *Momordica balsamina*

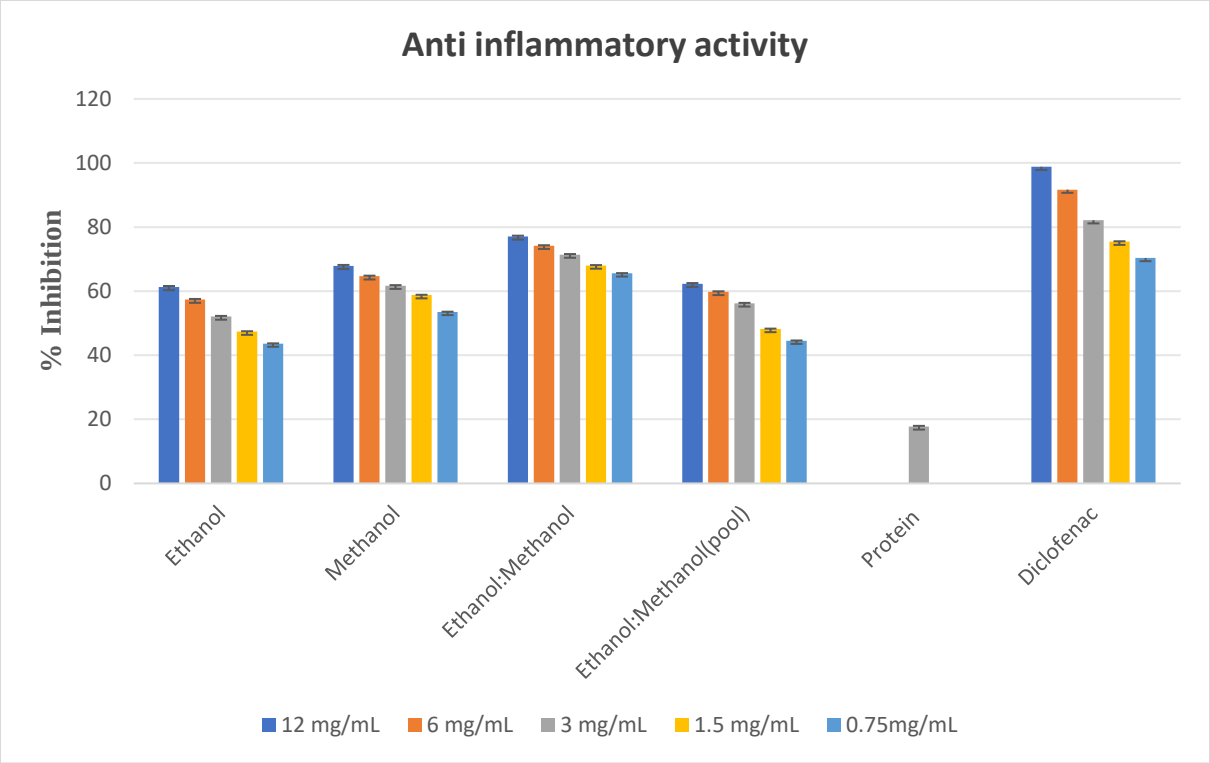


Figure 8: Anti Inflammatory activity of various extracts from *Momordica balsamina*

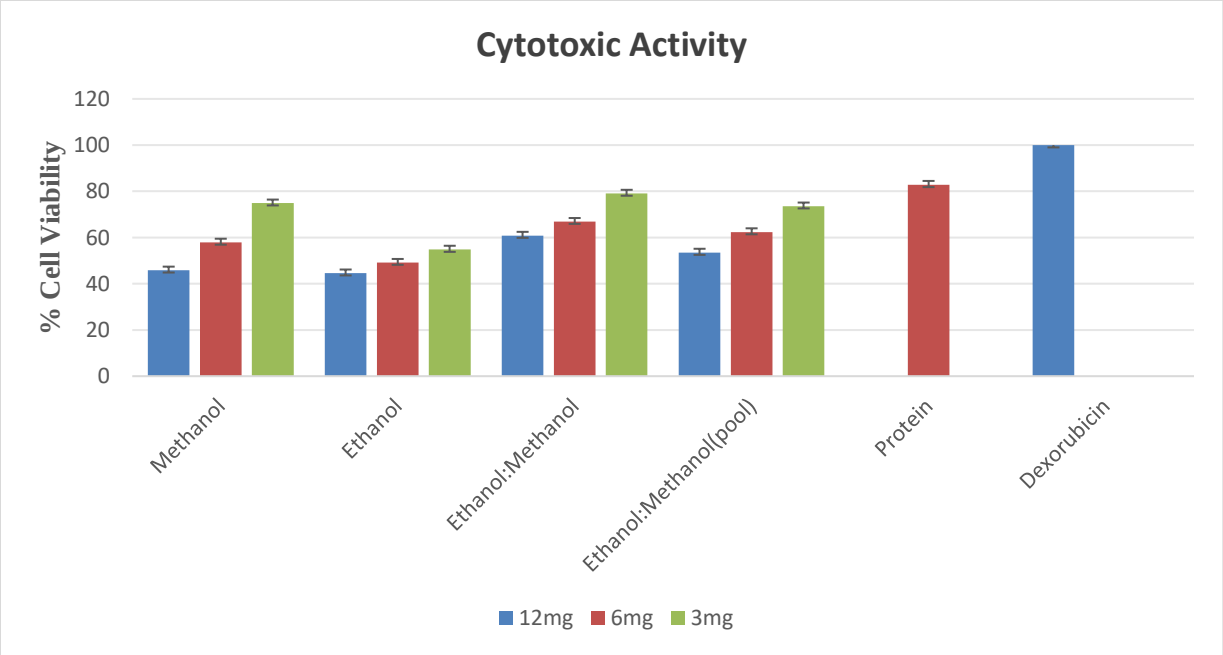


Figure 9: Cell Viability of solvent and protein extracts from *M. balsamina*

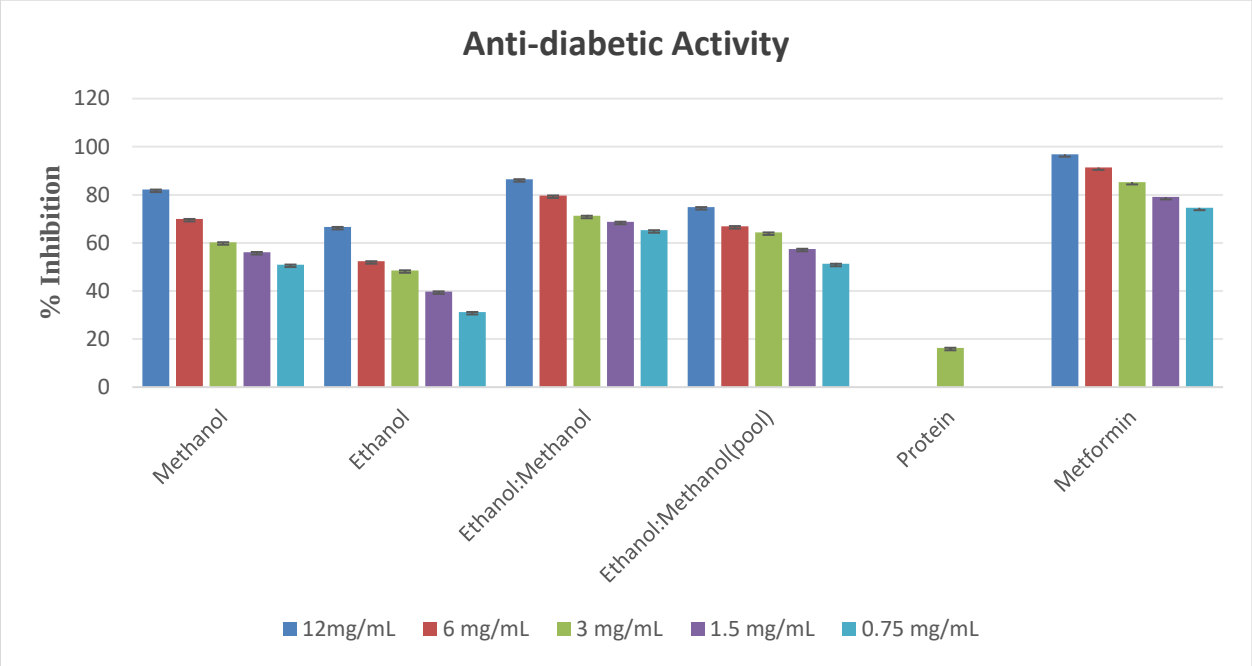


Figure 10: Antidiabetic activity of the various solvent and protein extracts from *Momordica balsamina*

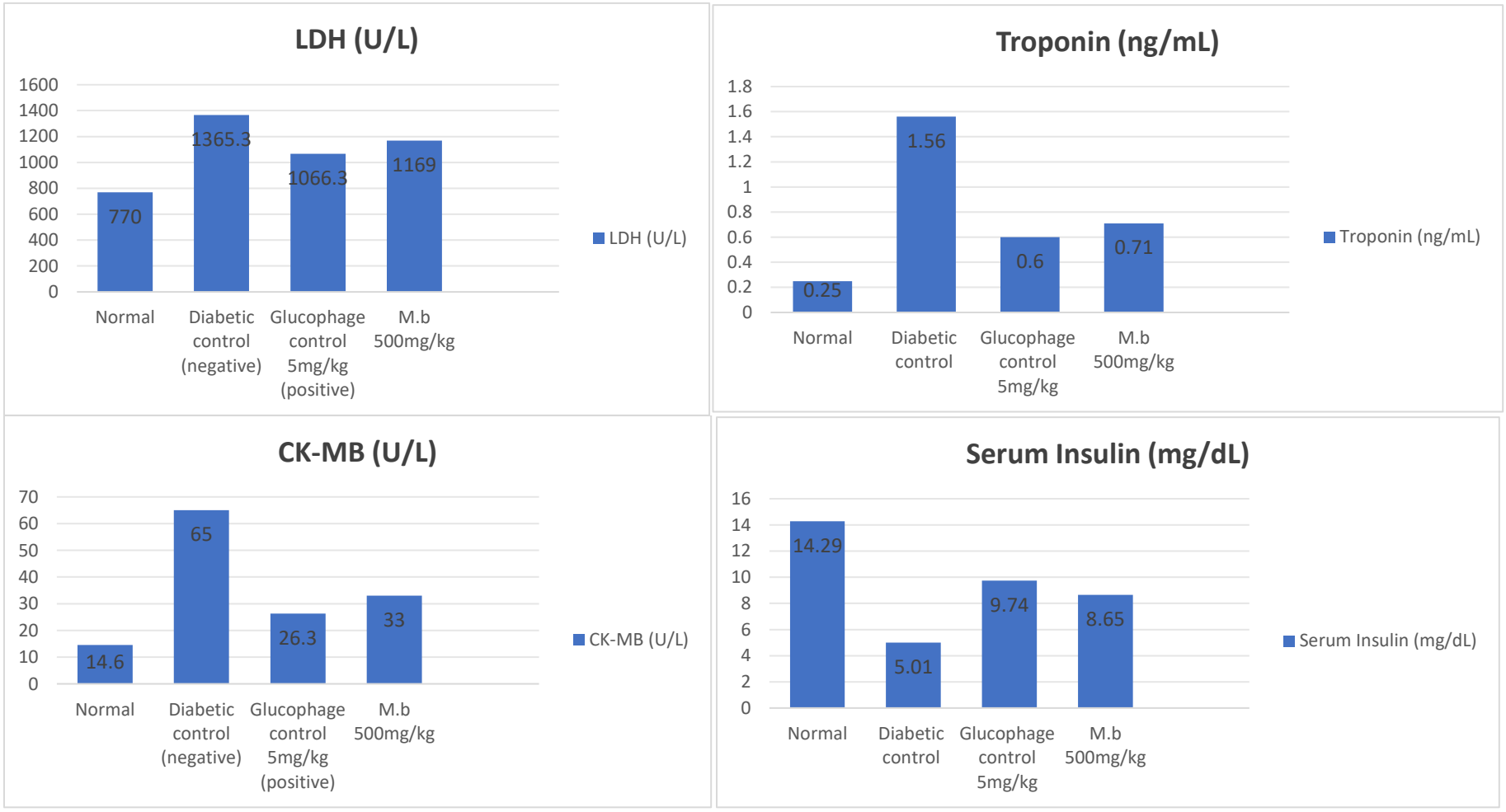


Figure 11: Graphs for effect of extracts on cardiac biomarkers and serum insulin

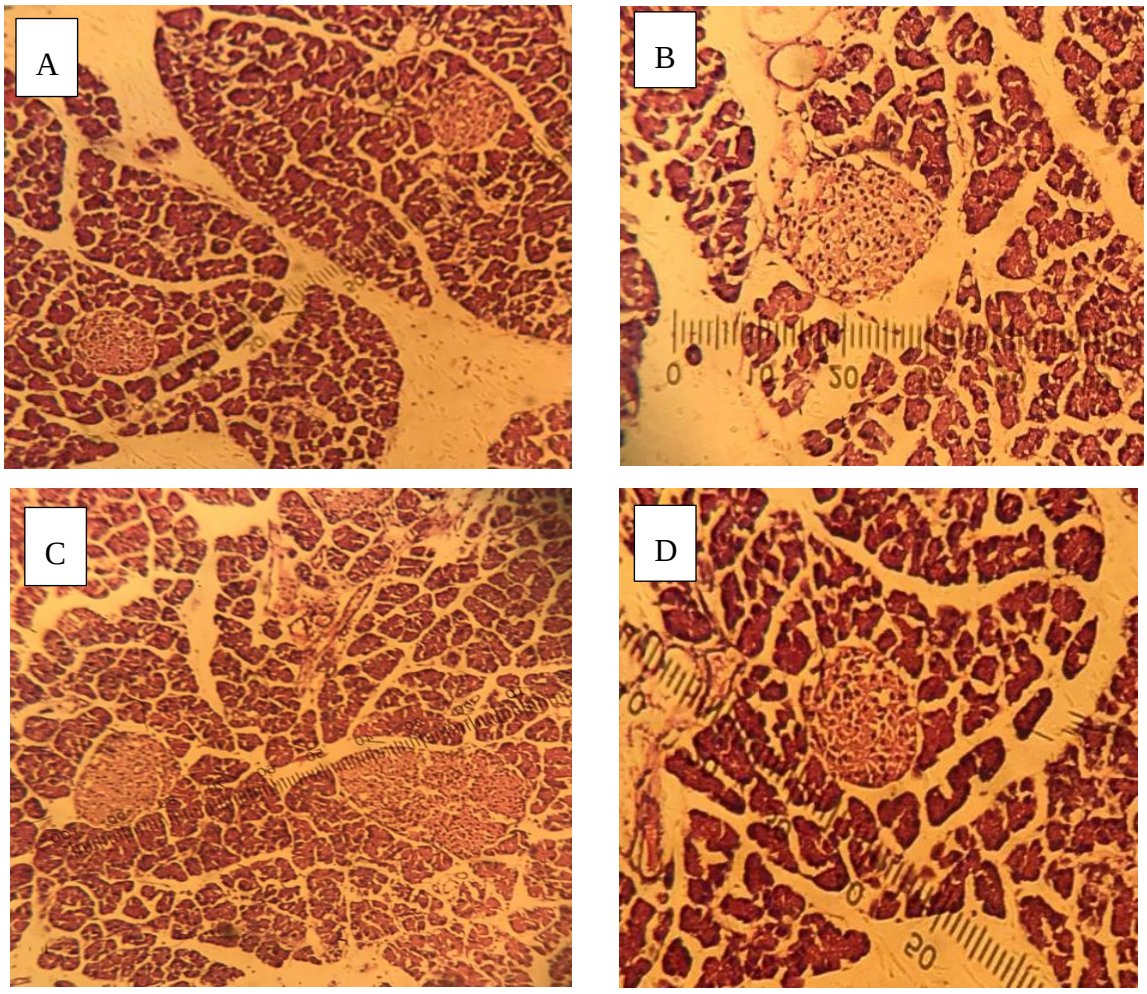


Figure 12: Histopathological analysis of Pancreas A) Normal pancreas of mouse. B) Pancreas of diabetic mouse with vacuolisation. C) Pancreas of Glucophage control with improvement in beta cells. D) Pancreas of experimental mouse adminitered with 500mg/kg *Momordica balsamina* extract, with improvement in beta cells and Islets of langerhans.

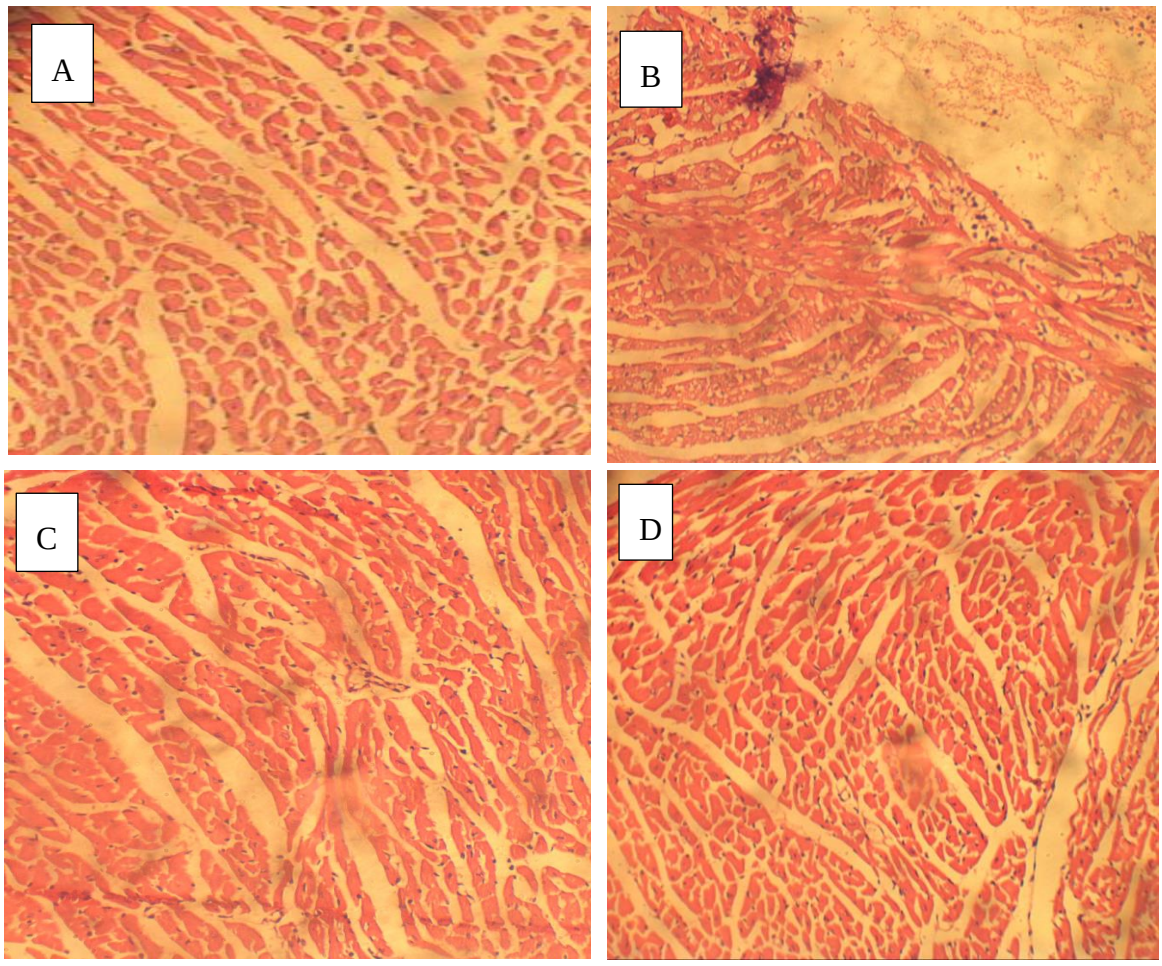


Figure 13: Histopathological analysis of Heart A) Normal heart architecture of mouse. B) Heart of diabetic control mouse with degenerating muscle fibres. C) Heart of glucophage control with reversal in cardiac injury. D) Heart of experimental mouse administered with 500mg/kg *Momordica balsamina* extract exhibiting improvement in cardiac myofibrils as compared to diabetic control.

Table 8: Identification and quantification of phenolic acids from fruit of *Momordica balsamina* as detected by HPLC-DAD.

S. No	Phenolic acids	*t_R (min)	Sample- ($mg.g^{-1}$)
1	Unknown	5.3	NI
2	Gallic acid	6.3	0.98 ± 0.05
3	2,4,6-trihydroxybenzoic acid	8.9	2.51 ± 0.51
4	Unknown	10.0	NI
5	Protocatechuic acid	12.4	1.01 ± 0.62
6	Protocatechualdehyde	14.6	3.34 ± 0.87
7	Sinapic acid	16.2	0.66 ± 0.08
8	2,4-Dihydroxybenzoic acid	17.9	1.54 ± 0.65
9	Hypogallic acid	19.6	12.11 ± 2.54
10	Caffeic acid	20.8	1.81 ± 0.91
11	Vanillin	22.8	3.01 ± 0.98
12	Syringic acid	23.9	4.87 ± 1.02
13	p-Hydroxybenzoic acid	25.6	1.23 ± 0.74
14	p-Coumaric acid	27.6	9.95 ± 1.87
15	Ferulic acid	29.7	1.02 ± 0.99
16	m-Coumaric acid	32.3	0.58 ± 0.05
17	o-Coumaric acid	35.5	5.57 ± 1.50
18	Unknown	39.8	NI
Total			50.19 ± 13.38

All analyses were mean of three experiments ± RSD.
N.I: Not Identified
*t_R= Retention time

Table 9. Separation and identification of phenolic acid standards with respective linearity & retention time by HPLC-DAD

S. No	Standards	$t_{R\ (min)}$	Regression equation	R^2	λ_{max} (nm)
1	Gallic acid	6.01	y = 305726x – 249684	0.999	227, 272
2	2,4,6-trihydroxybenzoic acid	9.22	y = 49119x + 29082	0.998	228, 255
3	Protocatechuic acid	12.06	y = 530511x + 112990	0.997	228, 259,294
4	Pyrogallolaldehyde	14.18	y = 337860x + 147020	0.999	234, 291
5	Protocatechualdehyde	14.35	y = 548015x + 303632	0.998	234, 281
6	Gentisic acid	14.92	y = 13444x - 1829.4	0.999	232, 327
7	Sinapic acid	16.26	y = 643555x - 1E+06	0.991	255
8	2,4-Dihydroxybenzoic acid	17.99	y = 200138x + 46398	0.998	255, 294
9	Hypogallic acid	19.61	y = 82657x – 14787	0.998	232, 314
10	Vanillic acid	20.19	y = 289390x – 82077	0.999	223, 260, 294
11	Caffeic acid	20.73	y = 169059x - 140031	0.996	233, 323
12	Vanillin	22.83	y = 626260x - 138097	0.999	233, 281
13	Syringic acid	24.03	y = 214749x – 72422	0.999	225, 275
14	*p*-Hydroxybenzoic acid	25.56	y = 88856x – 14995	0.999	234, 308
15	*p*-Coumaric acid	27.85	y = 213962x - 333316	0.995	232, 309
16	Chlorogenic acid	28.92	y = 97008x – 33773	0.998	233, 327
17	Ferulic acid	29.79	y = 174006x + 127640	0.999	235, 322
18	*m*-Coumaric acid	32.59	y = 533000x + 78590	0.999	232, 278
19	*o*-Coumaric acid	35.75	y = 7E+06x - 2E+06	0.999	232, 277
20	Cinnamic acid	45.05	y = 568487x + 305505	0.992	230, 280, 330

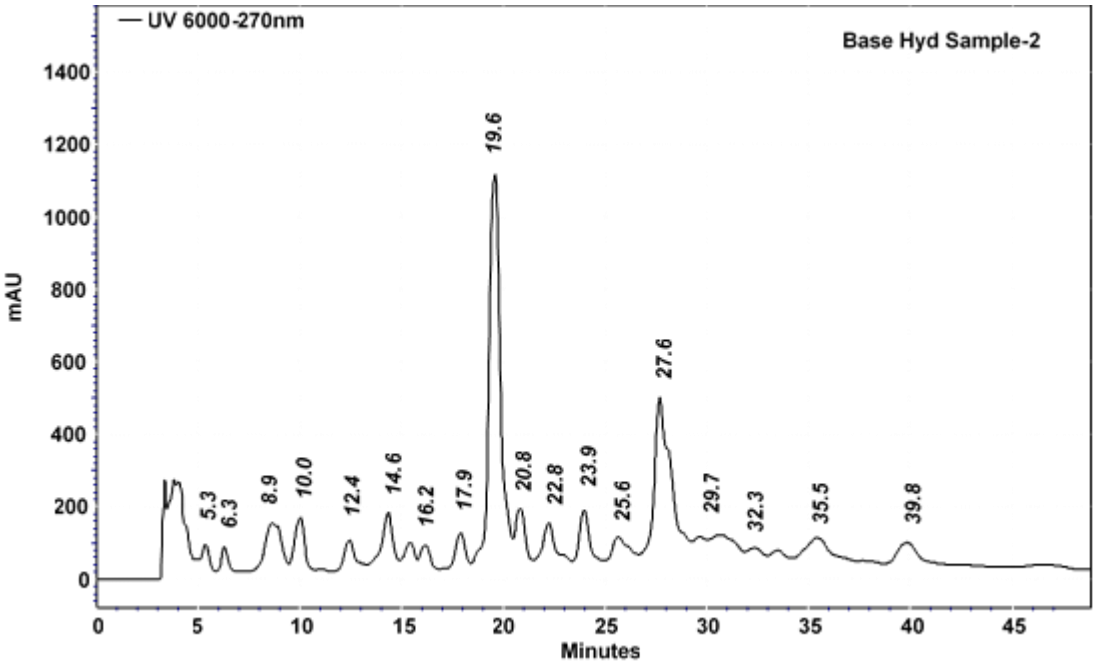


Figure 14: HPLC profile of phenolic acids extracted from simple sonication in base hydrolysis of fruit of *Momordica Balsamina*

3. Discussion

The LC-MS analysis showed the following compounds listed in the **Tables 2-5** and **Figures 3-6**. Solvent extracts were found to have various classes of compounds such as triterpenoids, flavonoids, coumaric acid derivatives, xanthones, quinolines, glycosides, and monoterpenoids. A study by Thakur (2009) also revealed that *M. balsamina* contain flavonoids, coumaric acid derivatives, xanthones, terpenes, glycosides, and saponins having diverse medical importance.

For determination of bound phenolic acids in fruit of *M. balsamina*, base hydrolysis ultrasound assisted extraction was performed. The HPLC results showed that 18 phenolic acids were detected, including 3 unknown phenolic acids (not identified) and three classes of compounds such as cinnamic acid derivatives, benzoic acid derivatives and aldehyde derivatives. High levels of hypogallic acid and p-Coumaric acid were seen **(Table 8)**. Our study findings are related to study reported by Khalid et al., (2021) on *Momordica Charantia*.

The antioxidant activity of *Momordica balsamina* is commonly attributed to its rich phytochemical composition, particularly its total phenolic content (TPC) and other bioactive compounds such as terpenoids and flavonoids. In our study, we compared the antioxidant activities of different extracts

of *Momordica balsamina* viz. ethanol, methanol, ethanol+methanol(1:1), pooled ethanol extract: methanol extract and crude proteins sample, by performing Total Phenolic Content assay, Total Flavonoid Content assay and DPPH assay. From the results, we inferred that methanol: ethanol (1:1) extract of *M. balsamina* exhibited the highest (40.31 GAE mg/g) TPC values (**Table 6)**, whereby the crude proteins extracted from the plant showed the lowest (7.42 GAE mg/g) content. Study conducted by Souda et al., (2018) suggested antioxidant activity of *M. balsamina* through total phenolic content (TPC) evaluation, founding it to be potent antioxidant plant. Similar trend was observed in TFC calculations, the highest TFC content (52.92 mg CE/g) in ethanol: methanol extract in case of *M. balsamina,* while the lowest range was observed in crude protein sample. In DPPH study, the highest percentage scavenging activity (62.42%) was observed by ethanol: methanol extracts in case of *M. balsamina* , whereby the lowest was exhibited by crude protein sample **(Figure 7)**. The optimal polarity spectrum is observed by mixed solvent system, which makes it possible to extract both polar and semi-polar phytochemicals, most commonly phenolics and flavonoid, that effectively scavenge free radicals. This synergy maximizes solubility of phenolics and flavonoid, which translates to higher DPPH radical scavenging activity, TFC and TPC. Overall, the antioxidant potential of *M. balsamina* make it a valuable candidate in research and medicine, although more studies are needed to fully elucidate its efficacy and related mechanisms in various contexts.

Various studies on the antibacterial activity of *M. balsamina* indicate that extracts of *M. balsamina* demonstrate significant inhibitory effects against selected bacterial strains. In this intended study, we chose two bacterial strains i.e *Escherichia coli* and *Streptococcuc spp.* Our results showed a remarkable growth inhibition by all different extracts, forming clear inhibition zones. The diameter of the zones varied among different solvent extracts, whereby ethanol extract exhibited the greatest zone of inhibition in case of *E. coli* and crude proteins showed the smallest (**Table 7**). In case of *Streptococcus spp.* the largest zone of inhibition was observed in ethanol extract, while the smallest zone by crude proteins sample.

All of the solvent extracts and protein extract showed antifungal activity against *Aspergillus flavus* and *Fusarium oxysporum,* using fluconazole as a standard drug (**Table 7**). The diameter of zone of inhibition was found in the range of 2.89-19.3 mm. Maximum zone of inhibition for *Aspergillus flavus* found as 18.10mm with ethanol+methanol(1:1) and lowest was 3.23mm with the crude protein extract. For *Fusarium oxysporum*, values were obtained in the range of 2.89mm to

19.30mm. Antibacterial and antifungal activities of these extracts are related to the bioactive compounds present in *M. balsamina* (Hassan, L. and K.J. Umar., 2006).

A significant anti-diabetic activity of *Momordica balsamina* has been evidenced by various studies. Our results reported that ethanol+methanol (1:1) extract exhibited the highest alpha amylase inhibition anti-diabetic activity (85.98 %) while the lowest activity was observed in crude protein sample **(Figure 10)**. Danyaya et al., (2023) demonstrated the significant antidiabetic effects of *M. balsamina* aqueous methanolic extract in diabetes-induced rats in a way that it reduces levels of blood glucose. Rao. P and G. Krishna (2017) studied the in-vitro alpha-amylase inhibition and in-vivo antidiabetic potential of *M.dioica* and revealed it to be a good antidiabetic agent.

The *in-vitro* studies have demonstrated significant anti-inflammatory activity of various plant extracts of *M. balsamina* and focus on their effects on inflammatory pathways and markers. In this study, we evaluated the BSA anti-inflammatory effects of various extracts of *M. balsamina.* Each extract showed potential anti-inflammatory effects by preventing the denaturation of BSA protein exposed to high temperature. Ethanol+methanol (1:1) showed the highest percent anti-inflammatory activity (85.56%). Crude protein sample of *M. balsamina* showed the lowest percent anti-inflammatory activity (17.52%) **(Figure 8)**. Mabasa et al., (2021) indicated potential anti-inflammatory effects of *M. balsamin* due to presence of bioactive compounds such as terpenoids and flavonoids.

The MTT cytotoxic activity of *Momordica balsamina* has been evaluated in various studies, suggesting its potential as potent anticancer plant. In this study, we found crude protein sample to possess the highest percentage viability (82.81%) whereby ethanol extract showed the lowest percentage of viable cells (44.6%) **(Figure 9)**. The low percent viability indicates the high cytotoxicity of extract or treatment and cells have killed or affected significantly, suggesting that the plant has ant cancerous effects against cancer cells. The lowest percent viability of ethanol extract indicates that this solvent extract has highest potential as anticancer agent and vice versa. In one of the studies conducted by Serala et al., (2021), it was suggested that *M. balsamina* exhibited potent cytotoxic effects against colorectal cancer cell lines by interfering with metastatic processes, leading to reduced cell viability.

Diabetic cardiomyopathy is an alarming concern in the diabetic patients, characterized by mitochondrial dysfunction, leading to structural and functional abnormalities in cardiac

architecture (Pan, X., et al., 2024). The bioactive compounds present in botanical sources show promise in managing diabetic cardiomyopathy by targeting the cellular mechanisms (Kotturu, S.K., et al., 2021). The therapeutic potentials of selected plant, *Momordica balsamina* in treating diabetic cardiomyopathy has not been explored previously. Given the highest efficacy observed *in-vitro,* we selected ethanol+methanol(1:1) for further *in-vivo* analysis, specifically targeting diabetic cardiomyopathy. We chose serum Insulin, LDH, CK-MB and Troponin I as markers to comprehensively evaluate metabolic and cardiac impacts of diabetic cardiomyopathy and the potentials of our plant in managing the disease and its symptoms. Selected plant showed improvement in serum insulin levels of diabetic mice administered regularly with 500mg/kg of plants extracts. LDH and CK-MB are the indicators of tissue damage and cardiac stress. The results exhibited by selected plant showed decline in the serum LDH and CK-MB levels as compared to diabetic control groups shown in **Figure 11**. Troponin I is a highly specific marker for myocardial injury, providing a detailed picture of cardiac health. Significant decline in Troponin I level was also observed in our treated groups. Moreover, histopathological analysis of heart and pancreas in diabetic, normal and treatment mice groups showed an improvement in the pancreatic and heart cells' architecture, suggesting the roles of selected plants in improving structural abnormalities of heart and pancreas **(Figures 12,13)**. The study indicates the protective effects of the selected plant extract on cardiac and pancreatic functions, suggesting a decrease in myocardial damage and enhanced secretion of serum insulin. The observed biochemical improvements underscore the potentials of *M. balsamina* in mitigating diabetes related complications, specifically in improving cardiac health and preserving glycemic controls in diabetic conditions. Our study correlates with the findings of Kulkarni et al., (2020) studied the progression of diabetic cardiomyopathy and its mitigation through *Bauhinia variegata* by studying various heart biomarkers, such as, CK-MB, LDH and lipid markers, concluding the significant effects of *Bauhinia variegata* in management of diabetic cardiomyopathy. Further studies and research on *M. balsamina* will explore the mechanisms involved in managing such conditions.

Conclusion

Our study's findings revealed the pharmacological potential of the selected plant from Sargodha region of Pakistan. Plants native to Pakistan have great pharmacological significance and are ideal candidates to use as medicine. More clinical studies and use of high-performance techniques for purification of bioactive compounds are advised for the researchers.

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