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Susceptibility of poultry associated bacterial pathogens to *Momordica charantia* fruits and evaluation of *in vitro* biological properties

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ABSTRACT

The emerging incidence of antibiotic resistance trait among the bacteria populating poultry presents a devastating public health issue. On the other hand, at present, diabetes and obesity are the most serious public health issues and are increasing subsequently at alarming rate. In view of this, the present *in vitro* context was aimed to investigate the antibacterial activities of *Momordica charantia* (*M. charantia*) fruits extracts against poultry associated *Bacillus* spp. and to assess further its phytoconstituents, alpha-(α)-glucosidase activities, and anti-obesity properties. The anti-pathogenic attributes of *M. charantia* fruit extracts were carried out using disc diffusion assay and results showed the pronounced antibacterial trait of ethanolic extract with maximum zone of inhibition of 28.3 ± 1.2 mm against *Bacillus licheniformis*. The qualitative phytochemical analyses of fruit extracts illustrated the presence of diverse phytoconstituents. The α-glucosidase inhibition assay for the extracts was performed according to the α-glucosidase activity kit. The results depicted the lowest α-glucosidase activity (57.13 ± 2.3 to 18.14 ± 1.3 U/L) in the presence of ethanolic extract at varied concentrations. The anti-obesity potentialities of fruit extracts were demonstrated in terms of porcine pancreatic lipase (PPL type II) activity using p-nitro-phenyl butyrate (p-NPB) as a substrate. The ethanolic extract of *M. charantia* fruits was observed to exhibit maximum inhibition of pancreatic lipase ranging from 20.12 ± 2.3 to 68.34 ± 1.3% in a dose dependent manner with an IC_{50} value of 607.6 ± 1.3 μg/mL. FTIR and GC-MS results indicated the presence of distinct compounds in the ethanol extract and major bioactive constituents were found to be Dimethyl sulfone (35.24%), 9-octadecanamide (20.52%), Pentadecanoic acid (6.64%), Lanost-9 (11)-en-18-oic acid, 23-(acetylxyl)-3-(4-bromobenzoyl) oxyl-20-hydroxyl-gamma-lactone (2.6%), and 2,2-sulfonyldiethanol (2.46%). In conclusion, *M. charantia* fruits could be of great concern in pharmaceutical industries due to its adequate biological properties and may also help in the management of poultry associated bacterial pathogens.

1. Introduction

Poultry associated bacteria are creating an alarming situation for public health due to the indiscriminate use of conventional antibiotics, thereby causing high emergence of antibiotic resistant strains. These bacteria can be transmitted from poultry to humans through the food chain with serious consequences [1]. This, therefore, necessitates a need for the identification of promising therapeutic agents. On the other hand, diabetes mellitus is one of the most common chronic metabolic disorders worldwide. This dreadful endocrinological syndrome represents a rise in the sugar level of the blood due to the appropriate production of insulin. India alone contains approximately 30 million counts with diabetes mellitus and the incidence will increase in next 10 years [2]. Surprisingly, antibiotics cause considerable alterations in the human intestinal flora and exposure to antibiotics has been linked with development of obesity and glucose homeostasis disturbances in patients with type 2 diabetes [3]. At present, the alarming increase in the obesity has become one of the world's most serious public health problems, estimating that 58% of world's populace will become obese in next 15–20 years [4]. The severe complications of high body fat include hyperlipidemia, hypertension, arteriosclerotic disease, cardiovascular disease, and most importantly diabetes [5].

Medicinal plants play a prominent role as alternative remedy for the maintenance of human health. Plant associated drugs due to their non-toxicity and lack of complications, are considered more effective when compared to the synthetic drugs commercially available in the market [6]. Vegetables, herbs, and spices are used not only for enhancing the flavour, colour, and aroma of food items but also for various medical

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purposes [7]. *Momordica charantia* (*M. charantia*), commonly called as 'bitter guord', is one of the medicinally important herbaceous plants that belong to the family Cucurbitaceae. It is one of the healthiest food items of tropical and sub-tropical regions and is used for the treatment of several ailments.

M. charantia is used for the treatment of diabetes in several parts of the world due to the presence of Gurmarin (a polypeptide) which has potential ability to regulate the sugar level [8]. The bioactive components of the plants exert their hypoglycaemic properties via various mechanisms such as stimulation of peripheral cell glucose utilization, intestinal glucose uptake inhibition, inhibition of gluconeogenic enzymes, stimulation of HMP pathway, and preservation of pancreatic cells [9]. Previous *in vivo* studies suggested that *M. charantia* could reduce body weight and visceral fat mass [10]. According to the study of Bano et al. [11] and Chen et al. [12], aqueous extract and seed oil supplementation of this plant significantly reduced body weight gain by cAMP activated protein kinase (PKA) mediated apoptosis in white adipose tissues of mice. Other mechanistic study of *M. charantia* includes reduced lipogenic gene expression in adipose tissues [13], peroxisome proliferator activating receptors (PPARs) [14], and increased lipid oxidation in adipose tissues [15].

In general, the vast medicinal properties of plants exist due to the bioactivity of phytoconstituents that contributes in the development of therapeutic agents against several human disorders. Several bioactive compounds such as momorchanins, momordicilin, momordicinin, momordenol, momordicius, momordolol, momordin, charantin, cryptoxanthin, charine, cucuritacins, cucurbitns, diosgenin, cucuritanes, erythrodiol, cycloartenols, elaeostearic acids, galacturonic acid, goyaglycosides, goyasaponins, gentisic acid, and multiflorenol are the major components of *M. charantia* [16]. Different parts of this plant such as leaves, roots, fruits, and seeds are widely used for the treatment of lumbago, ulceration, bone fracture, leprosy, haemorrhoids, jaundice, HIV, inflammation, leukemia, microbial infection, and tumor [17].

In the current scenario, there is a vast demand of therapeutic agents from natural sources not only to find effective alternative antibacterial agents against poultry associated pathogenic bacteria but also to overcome the complications of existing drugs and possible treatment of metabolic disorders globally. Considering the aforesaid potential medicinal properties of *M. charantia*, the present context was investigated to assess the anti-pathogenic trait of *M. charantia* fruit extracts against poultry associated bacteria. Further, the study was focused to determine the phytoconstituents, *in vitro* α-glucosidase activity, and anti-obesity characteristics of fruits of this herbaceous climbing plant.

2. Materials and methods

2.1. Sample collection

The fresh fruits of *M. charantia* were purchased from Green veggies market, Chennai, India. It was further authenticated by Dr. G. Jeya Jothi, Taxonomist, Department of Plant Biology and Biotechnology, Loyola College, Chennai, India.

2.2. Chemicals and reagents

All chemicals and reagents used in this investigation were of analytical grade and obtained from Sigma chemical company.

2.3. Extract preparation

The fresh *M. charantia* fruits were washed with distilled water in order to remove dust and other foreign particles. Fruits were sliced into thin layers and kept for shade drying for 2–3 days in the laboratory. The shed dried slices were blended into fine powder using sterile mortar and pestle and it was extracted sequentially by hot extraction method where ethanol, acetone, distilled water, chloroform, and petroleum ether were used as solvents. The dried powder was mixed well with respective solvents in the ratio of 1:4 and allowed for vigorous mixing. After 2 days of intermittent mixing, the mixture was filtered using Whatman filter paper. The filtrate was collected in a beaker and kept at 60–70 °C in order to evaporate the solvent and to obtain the solvent extracts of *M. charantia* fruits. Extracts were collected in the sterile eppendorf tubes for further phytochemical and therapeutic screening.

2.4. Qualitative phytochemical analysis

The identification of phytochemical constituents in different solvent extracts of *M. charantia* fruits was carried out according to the methodology of Harborne [18]. Extracts were analyzed for the presence of several bioactive phytochemicals such as tannins, saponins, flavonoids, alkaloids, glycosides, cardiac-glycosides, terpenoids, phenols, steroids, coumarins, anthocyanins, and betacyanin.

2.5. Bacteria of interest

Poultry associated pathogenic bacteria viz. *Bacillus licheniformis* (*B. licheniformis*; Accession-KC424492), *Bacillus subtilis* (*B. subtilis*; Accession-KC414759), *Bacillus mojavensis* (*B. mojavensis*; Accession-KC918877), *Bacillus cereus* (*B. cereus*; Accession-KY795954), and *Bacillus methylotrophicus* (*B. methylotrophicus*; Accession-KC424493) were collected from Department of Biotechnology, Loyola college, Chennai, India. All the bacterial cultures were sub-cultured in Nutrient broth medium (HiMedia) and stored in glycerol stock (50% v/v) at −80 °C for further experimental analyses.

2.6. Antibacterial assessment against poultry associated bacteria

Antibacterial activities of fruit extracts were determined according to disc diffusion method [19]. Each bacterial inoculum was prepared in 5 mL of Nutrient broth (HiMedia), adjusted to a 0.5 McFarland scale (1×10^6 CFU/mL), and incubated at 37 °C for 24 h in a rotatory shaker. After the required period of incubation, bacterial cultures were swabbed on Mueller Hinton Agar (HiMedia) plates. Subsequently, various extracts (25 μL) of fruits were transferred to sterile discs (6 mm) and allowed to soak for 10–15 min. The discs were transferred aseptically to the plates seeded with the respective *Bacillus* sp. with the help of ethanol dipped and flamed forceps, and incubated at 37 °C for 24 h. After 24 h of incubation, zone of inhibition (mm) formed by different extracts against the indicator bacteria were measured. Gentamicin (10μg/disc) was used as positive control and experiments were carried out in triplicate.

2.7. Relative percentage inhibition (RPI)

The RPI of fruit extracts against poultry associated *Bacillus* spp. was carried out according to the methodology of Gutiérrez-Morales et al. [20].

2.8. α-glucosidase activity

The α-glucosidase assay for extracts was performed according to α-glucosidase activity kit (SIGMA-ALDRICH). The assay is based on the kinetic reaction. The sample was prepared at different concentration (100–1000 μg/mL). Twenty microliters of distilled water was transferred to 96 well plate. In addition to this, 200 μL of water was added into each well. Similarly, 200 μL of Calibrator was added into separate well. Master reaction mix was prepared by adding 200 μL of assay buffer and 8 μL of α-NPG substrate for one well assay. Different concentrations of samples were transferred to separate wells of the plate and 200 μL of the master reaction mix was transferred to each of the sample wells. The content of wells was mixed uniformly by tapping. The initial absorbance was measured at 405 nm $(A_{405})_{initial}$. Samples

were incubated at room temperature for 20 min and the final absorbance was measured at the same wavelength $(A_{405})_{final}$. One unit of α-glucosidase is the amount of enzyme that catalyzes the hydrolysis of 1.0 μmol substrate per minute at pH 7.0. The α-glucosidase activity (U/L) of the sample was calculated as-

$$\frac{(A405)final - (A405)initial}{(A405)calibrator - (A405)water} \times 250 \quad (1)$$

2.9. Anti-obesity assay

Porcine pancreatic lipase (PPL type II) activity was measured using p-nitro-phenyl butyrate (p-NPB) as a substrate. The pancreatic lipase activity was calculated according to the methods of Zheng et al. [21] with slight modifications. The PPL stock solutions (1 mg/mL) were prepared in a 0.1 mM potassium phosphate buffer (pH 7.0) and solutions were stored at −20 °C. In order to determine the lipase inhibitory activity, extracts (100–1000 μg/mL) were pre-incubated with PPL for 1 h in potassium phosphate buffer. The reaction was then started by adding 0.1 μL NPB as a substrate, all in a final volume 100 μL. After incubation at room temperature for 5 min, the amount of p-nitrophenol released was measured by reading the absorbance at 405 nm using UV–Vis spectrophotometer. The activity of the negative control was also calculated with and without inhibitor.

The inhibitory activity was calculated according to the formula given below:

$$\text{Lipase inhibitory activity (\%)} = 1\text{- } [(B\text{-}b)/(A\text{-}a) \times 100] \quad (2)$$

Where, 'A' is the activity without the inhibitor and 'a' is the negative control without the inhibitor. 'B' is the activity with inhibitor and 'b' is the negative control with inhibitor. Dimethyl sulfoxide (DMSO) was used as negative control and its activity was also examined.

2.10. Analytical assay

2.10.1. Thin layer chromatography (TLC)

Among various solvent extracts analyzed for biological activities, ethanolic extract of *M. charantia* fruits exhibited promising therapeutic properties, and thus, ethanolic extract was chosen for analytical studies. TLC was performed on aluminium plates coated with silica gel, which were cut from the original plates into 8 × 3 cm before use. About 5–10 mg of the dried extract was dissolved in DMSO and mixed well. A spot was marked 2 cm from the bottom of the plate and the sample was loaded using a capillary tube and left for drying. Acetone and water (9:1) were used as mobile phase. After the run, the dried plates were then viewed under UV light at 302 and 325 nm, and in iodine chamber. Rf values were calculated in order to observe the bioactive metabolites present in the extract.

2.10.2. Fourier transform infra-red (FT-IR) spectroscopy analysis

Three milligrams of the crude ethanolic extract were mixed with 300 mg of KBr and pressed into a pellet. The pellet was placed into the sample holder and FT-IR spectra were recorded in the range of 4000–450 cm^{-1}in FT-IR spectroscopy [Model no.- IRAffinity-1(SHIMADZU)].

2.10.3. Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was carried out on a PerkinElmer Turbo Mass Spectrophotometer (Norwalk, CTO6859, and USA). Helium was used as carrier gas at a flow rate of 0.5 mL/min. One microlitre of the sample was injected and the inlet temperature was set at 250 °C. The oven temperature programme was set initially at 110 °C for 4 min, and then increased up to 280 °C. Total run time was set for 90 min. The quantification of components in crude ethanolic extract was done by relative peak areas calculation. Relative peak areas were calculated by dividing the peak area for compound by the total peak areas for the entire compounds detected and expressing this value as percent.

2.11. Statistical analysis

All the data of the experiments were carried out in triplicate and expressed as mean ± SD.

3. Results

3.1. Phytochemical screening

The phytochemical screening revealed the presence of diverse phytoconstituents in the ethanolic, aqueous, acetone, chloroform, and petroleum ether extracts of *M. charantia*. The study depicted the presence of tannins, saponins, quinones, flavanoids, alkaloids, glycosides, cardiac-glycoside, terpenoids, phenols, steroids, coumarins, and betacyanin in the ethanolic extract of the plant. Acetone extract was found to be rich in saponins, quinones, cardiac-glycoside, terpenoids, phenols, steroids, and betacyanin. On the other hand, aqueous and chloroform extracts showed the presence of few phytoconstituents viz. quinones, cardiac-glycoside, terpenoids, phenols, steroids, and betacyanin. In contrary to other solvent extracts, petroleum ether extract revealed the presence of saponins and terpenoids only (Table 1).

3.2. Antibacterial activity against poultry isolates

Solvent extracts of *M. charantia* fruit showed pronounced antibacterial activities against poultry associated bacteria (Table 2). The ethanolic extract showed potent bactericidal activity against *B. licheniformis* with maximum zone of inhibition of 28.3 ± 1.2 mm. Petroleum ether extract exhibited the lowest activity against all the indicator pathogenic bacteria with minimum zone of inhibition of 10.1 ± 1.3 mm against *B. cereus*. Other extracts showed antibacterial activities in the order of aqueous (15.3 ± 1.3–20.3 ± 1.2 mm) > acetone (14.3 ± 1.1–19.4 ± 1.3 mm) > chloroform (13.4 ± 1.1–16.3 ± 1.2 mm). In accordance to the bactericidal zone of fruit extracts, the RPI values were found to be affected (Table 2).

3.3. α-glucosidase activity

The α-glucosidase activity of solvent extracts such as ethanol, aqueous, acetone, chloroform, and petroleum ether extracts were estimated and results are summarized in Table 3. The ethanolic extract showed the lowest production of α-glucosidase in a concentration dependent manner, thereby indicating activity ranging from 57.13 ± 2.3

Table 1
Phytochemical screening of different solvent extracts of *M. charantia* fruits.

Tests	Ethanol extract	Aqueous extract	Acetone extract	Chloroform extract	Petroleum ether extract
Tannins	+	-	-	-	-
Saponins.	+	-	+	-	+
Quinones.	+	+	+	+	-
Flavonoids	+	-	-	-	-
Alkaloids.	+	-	-	-	-
Glycosides	+	-	-	-	-
Cardiac-glycoside	+	+	+	-	-
Terpenoids	+	+	+	+	+
Phenols	+	+	+	-	-
Steroids	+	+	+	+	-
Coumarins	+	-	-	-	-
Anthocyanins	-	-	-	-	-
Betacyanin	+	+	+	-	-

(+) = positive, (−) = negative.

Table 2
Antibacterial activity of various solvent extracts of *M. charantia* fruits against poultry associated *Bacillus* spp.

Bacteria	Extracts										Gentamicin
	Ethanol		Aqueous		Acetone		Chloroform		Petroleum ether		
	ZOI (mm)	RPI	ZOI (mm)	RPI	ZOI (mm)	RPI	ZOI (mm)	RPI	ZOI (mm)	RPI	ZOI (mm)
B. licheniformis	28.3 ± 1.2	ND	18.3 ± 1.2	ND	16.3 ± 1.2	ND	14.3 ± 1.4	ND	12.6 ± 1.2	ND	NA
B. subtilis	24.5 ± 1.3	80.8	15.3 ± 1.3	50.4	14.3 ± 1.1	47.2	15.3 ± 1.3	50.5	14.3 ± 1.4	47.2	30.3 ± 1.3
B. mojavensis	22.3 ± 1.2	78.8	16.5 ± 1.3	58.3	16.3 ± 1.2	57.6	16.3 ± 1.2	57.6	13.3 ± 1.3	46.9	28.3 ± 1.4
B. cereus	20.4 ± 1.1	ND	18.1 ± 1.1	ND	17.1 ± 1.1	ND	13.4 ± 1.1	ND	10.1 ± 1.3	ND	NA
B. methylotrophicus	21.3 ± 1.2	87.6	20.3 ± 1.2	83.5	19.4 ± 1.3	79.8	14.8 ± 1.1	60.9	12.3 ± 1.1	50.7	24.3 ± 1.3

Values are mean of experiments performed in triplicate and data are expressed as mean ± SD; ZOI – Zone of inhibition; mm – millimetre; ND – Not determined; NA – No activity.

Table 3
α-glucosidase activities and lipase inhibition properties of *M. charantia* fruits extracts.

Extracts (μg/mL)	α-glucosidase activity (U/L)	Lipase inhibition (%)	IC_{50} value (μg/mL) for lipase inhibition
Ethanol			607.6 ± 1.3
100	57.13 ± 2.3	20.12 ± 2.3	
250	47.24 ± 1.6	34.14 ± 1.6	
500	36.43 ± 1.5	46.45 ± 1.5	
750	28.12 ± 1.2	60.14 ± 1.2	
1000	18.14 ± 1.3	68.34 ± 1.3	
Acetone			852.3 ± 1.1
100	68.54 ± 1.4	17.34 ± 1.4	
250	61.85 ± 1.3	26.35 ± 1.3	
500	48.83 ± 1.3	34.73 ± 1.3	
750	37.54 ± 1.4	46.64 ± 1.4	
1000	26.13 ± 1.3	56.14 ± 1.3	
Aqueous			Not determined
100	68.34 ± 1.3	13.34 ± 1.3	
250	62.64 ± 1.3	22.64 ± 1.3	
500	50.84 ± 1.2	30.84 ± 1.2	
750	42.14 ± 1.4	39.14 ± 1.4	
1000	28.14 ± 1.3	41.14 ± 1.3	
Chloroform			Not determined
100	71.34 ± 1.3	10.14 ± 1.3	
250	65.64 ± 1.3	18.34 ± 1.3	
500	50.84 ± 1.2	24.64 ± 1.2	
750	46.14 ± 1.4	31.14 ± 1.4	
1000	41.14 ± 1.3	33.24 ± 1.3	
Petroleum ether			Not determined
100	88.34 ± 1.3	4.34 ± 1.3	
250	80.64 ± 1.3	12.44 ± 1.3	
500	74.84 ± 1.2	18.34 ± 1.2	
750	71.14 ± 1.4	23.14 ± 1.4	
1000	53.14 ± 1.3	26.14 ± 1.3	

Values are mean of experiments performed in triplicate and data are expressed as mean ± SD.

to 18.14 ± 1.3 U/L at lower (100 μg/mL) and higher concentrations (1000 μg/mL), respectively. The acetone extract revealed α-glucosidase activity of 68.54 ± 1.4 to 26.13 ± 1.3 U/L at 100 and 1000 μg/mL, respectively. In like manner, aqueous and chloroform extracts showed less potency towards α-glucosidase inhibition and depicted activity of 28.14 and 41.14 U/L, respectively at higher concentration. On the other hand, petroleum ether extract was found to be the least active in terms of α-glucosidase inhibition characteristic, and thereby α-glucosidase activities of 88.34 ± 1.3 and 53.14 ± 1.3 U/L were estimated at lower (100 μg/mL) and higher (1000 μg/mL) concentrations, respectively.

3.4. Anti-obesity property

Table 3 shows anti-obesity properties of various solvent extracts of *M. charantia* in terms of lipase inhibition. The ethanolic extract of *M. charantia* depicted maximum lipase inhibition property in a comparison with acetone, aqueous, chloroform, and petroleum ether extracts. The ethanolic extract showed increased lipase inhibition from 20.12 ± 2.3 to 68.34 ± 1.3% in a dose dependent manner with an IC_{50} value of 607.6 ± 1.3 μg/mL. On the other hand, acetone extract showed lipase inhibition ranging from 17.34 ± 1.4 to 56.14 ± 1.3% at 100 and 1000 μg/mL, respectively with an IC_{50} value of 852.3 ± 1.1 μg/mL. Similarly, aqueous extract and chloroform extract revealed the lipase inhibition properties of 41.14 ± 1.3 and 33.24 ± 1.3%, respectively at higher concentrations (1000 μg/mL). Petroleum ether extract was observed to be the least effective, showing lipase inhibition ranging from 4.34 ± 1.3 to 26.14 ± 1.3% in a concentration based manner.

3.5. Thin layer chromatography

The ethanolic extract showed most number of separation and reported the presence of three major components with Rf values of 0.27, 0.56, and 0.64 as visualized under iodine chamber and UV light (Figure not shown). The larger Rf value of the component indicates the larger distance travelled on the TLC plate.

3.6. FT-IR spectroscopy

The strong absorption peaks at 3745.76 cm^{-1} and 3387.00 cm^{-1} were obtained due to N–H and –OH stretching of acid. Absorption peaks at 2931.80 cm^{-1}, 2872.01 cm^{-1}, and 2856.58 cm^{-1} are mainly due to $–CH_3$ and $–CH_2$ aliphatic stretching. Peaks ranging from 2393.66 cm^{-1} to 2073.48 cm^{-1} are representative for asymmetric N–H as well as torsional stretching. On the other hand, absorption band obtained at 1734.01 cm^{-1} and 1649.14 cm^{-1} are representative for C═O carbonyl stretching of acids. Strong absorption peaks at 1543.05 cm^{-1} and 1519.91 cm^{-1} are because of NH_2 or N–H bonding. Peaks from 1454.33 cm^{-1} to 1323.17 cm^{-1} and 1244.09 cm^{-1} to 983.70 cm^{-1} are representative for C═O and C–N stretching, respectively (Fig. 1).

3.7. GC-MS analysis

The GC-MS chromatograph for the ethanolic extract of *M. charantia* is shown in Fig. 2. Table 4 shows the presence of diversified chemical constituents in the ethanolic extract of *M. charantia* fruits that includes bioactive compounds with their retention time and area percentage. Results showed that the extract was complex mixture of 24 bioactive compounds; many of which were present in trace amounts. On the other hand, Dimethyl sulfone (35.24%), 9-octadecanamide (20.52%), Pentadecanoic acid (6.64%), Lanost-9 (11)-en-18-oic acid, 23-(acetylxyl)-3-(4-bromobenzoyl) oxyl-20-hydroxyl-gamma-lactone (2.6%) and 2,2-sulfonyldiethanol (2.46%) were identified as the versatile compounds present in the ethanolic extract, and their respective structures are shown in Fig. 3.

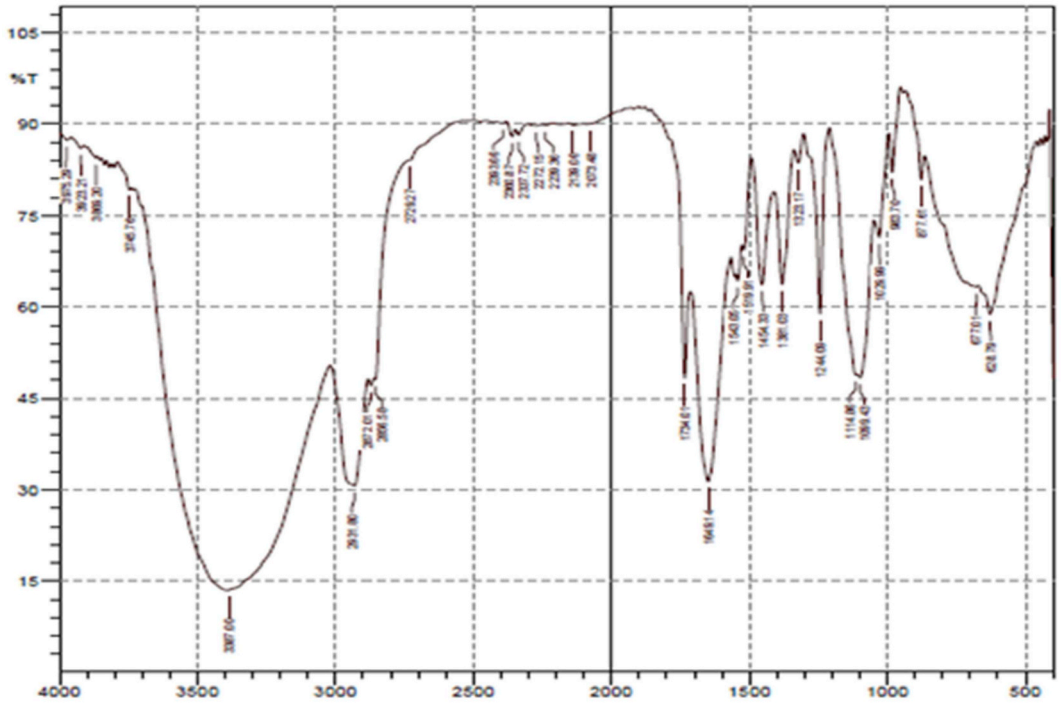

Fig. 1. FT-IR spectra for ethanolic extract of *M. charantia* fruits.

4. Discussion

In the current scenario, the significance of *M. charantia* in traditional medicine for several ailments has gained immense interest among researchers globally. In fact, the vast medicinal importance of plants solely depends on the bioactive chemical constituents, called as phytochemicals that produces physiological influences on the human health. Generally, investigating the diversiform chemical constituents of the medicinal plant are essential not only for its therapeutic applications but also as precursors for synthesizing complex chemical substances.

The present context contributes valuable information of desirable phytoconstituents in *M. charantia* fruit extracts. The qualitative analysis of fruit extracts revealed the presence of varied bioactive phytochemicals viz. tannins, saponins, quinones, flavanoids, alkaloids, glycosides, cardiac-glycoside, terpenoids, phenols, steroids, coumarins, and betacyanin in various solvent extracts, estimating in the order of ethanol > acetone > water > chloroform > petroleum ether. The presence of phytoconstituents more or less similar to our reports was observed by Singh et al. [22] and Ullah et al. [23] who analyzed the presence of similar kind of phytochemicals in various solvent extracts of *M. charantia* fruits. In fact, these phytochemicals are presumed to be secondary metabolites which are reported to exert a wide range of biological activities on physiological systems [24]. In addition, in the present study, the availability of promising phytoconstituents in varied extracts of *M. charantia* fruits provide a preliminary support for the traditional applications of this plant as a biotherapeutic agents.

Currently, conventional antibiotics have improved the performance of poultry economically [25] but the regular use of antimicrobials has caused the emergence of antibiotic-resistance bacterial strains. In the present study, a significant attempt was undertaken to determine the sensitivity of poultry bacterial pathogens to *M. charantia* fruit extracts. The findings revealed the effectiveness of *M. charantia* fruit extracts by inhibiting the growth of poultry associated bacteria, thereby suggesting the substantial use of *M. charantia* fruits as therapeutics against *Bacillus* spp. threatening public health through poultry.

At present, diabetes has become devastating threat to humankind. The quest for active, non-toxic, and cost-effective hypoglycaemic agents from natural sources, especially medicinal plants is a significant approach. The modern research has already conferred the broad-spectrum hypoglycemic activity of *M. charantia*. Chemical constituents isolated from *M. charantia* have manifested insulin-like effect [26]. In general, the hypoglycemic effect of *M. charantia* is achieved by reducing hepatic gluconeogenesis, increasing the synthesis of hepatic glycogen, and increasing glucose oxidation around red blood cells and fat cells [27]. To explore further the anti-diabetic potency of this valuable plant, we estimated *α*-glucosidase activity, a key enzyme known to catalyze carbohydrates into glucose. In fact, the glucose level in the blood can be controlled and maintained up to normal ranges by inhibiting α-glucosidase [28].

The outcomes of the present investigation provide a significant step towards the inhibition of α-glucosidase in a dose dependent manner, the ethanolic extract being the most active. The α-glucosidase inhibitory property of ethanolic extract may be due to the presence of glycoside in it. Glycosides consist of sugars that may be structurally similar to carbohydrate which is a substrate of α-glucosidase [29]. The α-glucosidase inhibition characteristic of ethanolic extract was found to be greater than those of acetone, water, chloroform, and petroleum ether because its active constituents may have a greater synergistic response towards α-glucosidase inhibition. Moreover, the greater α-glucosidase inhibitory characteristic of ethanol extract of *M. charantia* fruits may be due to the availability of higher phenols and flavonoids. Interestingly, Joseph and Jini [9] reported that the hypoglycamic action of *M. charantia* is possible due to alkaloids, insulin like peptides, and a mixture of steroidal sapogenins known as charantin. Besides the above mentioned statements, *M. charantia* exerts hypoglycaemic properties through various physiological and biochemical mechanisms viz. stimulation of peripheral and skeletal muscle glucose utilization [30,31], inhibition of adipocyte differentiation [32], stimulation of key enzymes of HMP pathway [27], and preservation of islet cells [33]. Surprisingly, *M. charantia* is known to contain a high dosage of 'plant insulin' and lowers the blood-sugar levels significantly [34]. In fact, 'plant insulin' is an analogue to animal insulin existing in plants [35]. Polypeptide-p is an unidentified insulin-like protein similar to bovine insulin found in *M. charantia* fruit [36].

In the last few decades, obesity has become epidemic life-style

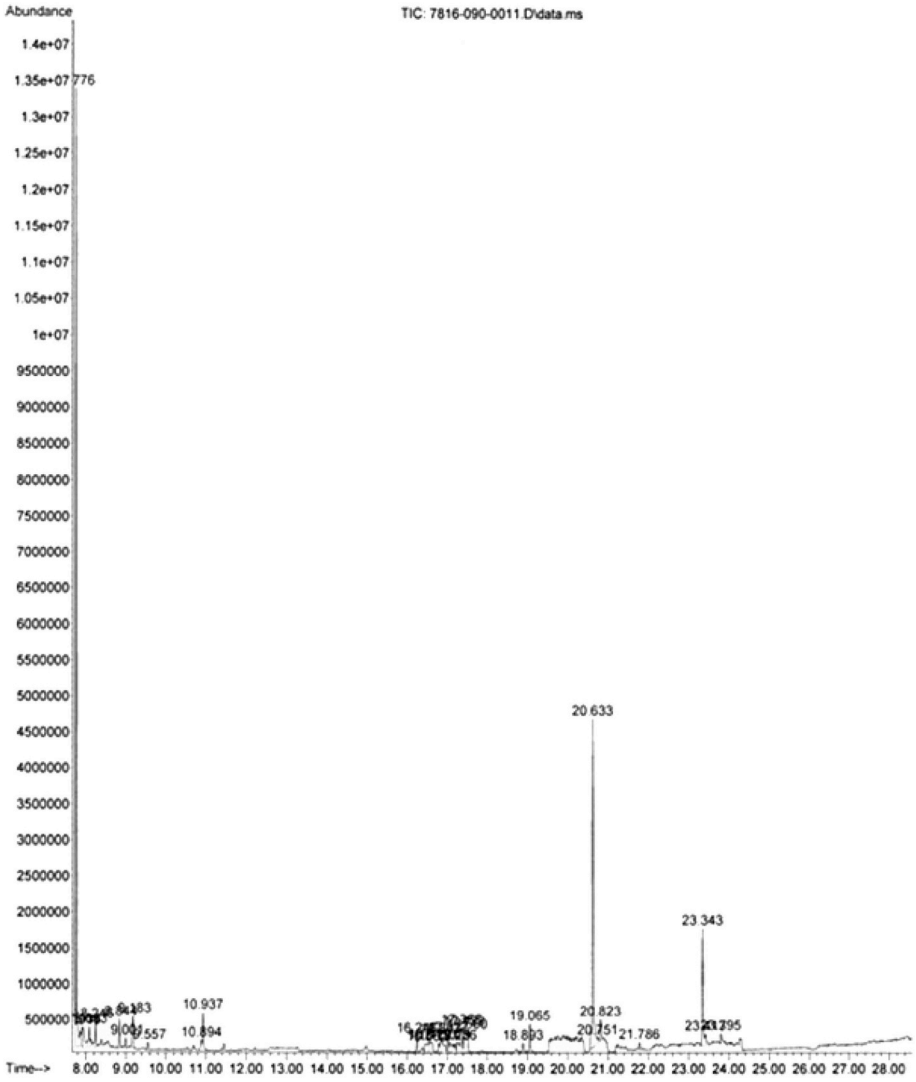

Fig. 2. GC-MS chromatogram for ethanolic extract of *M. charantia* fruits.

disorder in developed as well as developing countries. Herbal medicines have been exploited for therapeutic benefits in order to treat obesity and obesity associated metabolic disorders. In fact, body weight gain and abdominal fat deposition are the early symptoms of obesity. *In vivo* studies had already depicted the potentiality of *M. charantia* extract to reduce body weight in high fat diet induced obesity in laboratory animals. This weight reduction may be a result of increased fatty acid oxidation which ultimately facilitates weight reduction [37]. Similarly, *M. charantia* was observed to reduce the weights of epididymal white adipose tissue, visceral fat, and the adipose leptin and resistin mRNA levels in C57BL/6J mice fed with a high-fat diet [38]. In another report, a constant dose of aqueous extract of *M. charantia* significantly reduced body weight gain in rats [11].

In general, the pancreatic lipase is the key enzyme involved in the initiation of lipid digestion in the duodenum. Pancreatic lipase removes fatty acids from α and α′ positions of dietary triglycerides, thereby releasing β-monoglyceride and long chain saturated and polyunsaturated fatty acids. Thus, the inhibition of pancreatic lipase is one of the targeted approaches to identify efficacious anti-obesity agents for the therapy. In view of the crucial role of pancreatic lipase in the metabolisms of lipid, the current study was investigated to determine the potent role of diverse solvent extracts of *M. charantia* towards lipase inhibition. In this *in vitro* investigation, the concentration dependent ethanolic extract of *M. charantia* fruits was observed to be the most effective anti-obesity agent in terms of inhibiting pancreatic lipase. Our findings were in complete agreement with the reports of Sahib et al. [39] who evaluated *in vitro* anti-pancreatic lipase activity of ethanolic extract of *M. charantia* fruits at various concentrations and observed significant inhibition of pancreatic lipase. In the present context, the potential anti-lipase activity of ethanolic extract of *M. charantia* might be due to the presence of predominant amount of phytoconstituents, especially phenols and flavonoids. Previously, Ardevol et al. [40] suggested that the anti-obesity properties are attributed to the synergistic action of several flavonoids rather than a single flavonoid. Apart from this, in recent years, several studies reported *in vitro* anti-lipase activity of plethora of medicinal plants other than *M. charantia*, and suggested promising pancreatic lipase inhibition [41–43].

TLC analysis confirmed the presence of major bioactive component in the ethanolic extract of *M. charantia* fruits based on varied Rf values. Further, FT-IR spectrum was used to assess the stability of chemical constituents and the presence of diverse functional groups in the ethanolic extract of fruits. The present investigation depicted the presence of different peaks in the extract which was supposed to be obtained through stretching and bending vibrations in the region of infrared radiation. Additionally, different peaks were obtained due to the shifts in the FT-IR spectra too. GC-MS analysis of the ethanolic extract of *M. charantia* fruits showed predominant availability of bioactive components in the decreasing order of Dimethyl sulfone (35.24%) > 9-octadecanamide

Table 4
GC-MS analysis of ethanol extract of *M. charantia* fruits.

Peak	Retention time	Area (%)	Compound name
1.	7.779	35.24	Dimethyl sulfone
2.	7.913	0.98	Methanesulfinothoic acid, S-1propyl ester 1,5-heptadiene-3-yne (s)-(+)-1,2-propanediol
3.	8.091	1.61	Azetidine,1-chloro-2-propanol,1-chloro-3-(octylsulfinyl)-ethanol,2-(2-chloro-ethylthio)
4.	8.247	1.58	s-methyl methanethiosulphonate methyl 2-hydroxyethyl sulfoxide propyleneglycol
5.	9.005	0.49	Butane,1-(methlthio)-2,4—dithiapentane Butanitrile, 4-(methylthio)
6.	9.54	0.53	2,2-sulfinyldiethanol, 1-ethanol, 2-(ethylsulfinyl)-1-(2- hydroxylthio)-2-(vinylthio)-ethane
7.	10.891	0.85	1-ethanol, 2-(ethylsulfinyl)-ethanol, 2,2-sulfonylbis-1-propene.
8.	10.936	2.46	2,2-sulfonyldiethanol
9.	16.247	1.14	Propanoic acid, methyl ester, ethane
10.	16.500	1.53	Carbonic acid, 2-chloroethyl 2,2,2-trichloroethyl ester, 2-chloroethyl ester, bis(.beta.-chloroethyl) sulfoxide, propanoic acid
11.	16.545	0.76	Dibenzo (b.N)-30-10
12.	16.790	0.58	Fluchloralin Ethane, 1,1-dichloro-2-(-(2- chloro-ethoxy)-ethoxyl phenol
13.	16.842	1.21	Propane, 2-chloro-carbonochloridic acid, 1,6-hexanediyl eter, 2,2-dichloroethyl propyl carbonate
14.	17.079	1.04	n-hexadeconoic acid, dodeconoic acid, alpha-D-galactopyranoside
15.	17.079	1.22	Copper, 1,1-dichloro-5-methoxybenzofurazan, bis(4-bromo −3,5-cycloheadiene-1,2-dione
16.	17.235	0.58	Ethane, 1,1-dichloro-methyl 2,2,4-trichloro-3-oxypentanoate
17.	17.354	2.60	Lanost-9 (11)-en-18-oic acid, 23-(acetylxyl)-3-(4-bromobenzoyl)oxyl-20-hydroxyl-gamma – lactone
18.	18.892	0.71	1,3,2-oxyathiaborole, 2-ethyl-12-bromododecanoic acid, D-mannoheptulose
19.	19.063	1.88	Hexadecanamide, Octanamide, Undecaamide
20.	20.630	20.52	9-octadecanamide
21.	20.824	1.43	Octadcanamide, hheptanamid, 4-ethyl-5-methyl-7-nonenamide
22.	21.789	0.51	Propanoic acid, 2-chloro-2-propenyl ester Butyl pentyl carbonate (4-carbomyl-2-nitrophenyl)acetic acid
23.	23.342	6.64	Pentadecanoic acid
24.	23.795	0.76	Chlorfenapyr-7-octen-2-ol, 2,6-methyl-2,2,3,3,5,8,11-heptamethyl-4,7,10,13-tetraoxa-3-silaheptadcane

(20.52%) > Pentadecanoic acid (6.64%) > Lanost-9 (11)-en-18-oic acid, 23-(acetylxyl)-3-(4-bromobenzoyl)oxyl-20-hydroxyl-gamma-lactone (2.6%) > 2,2 sulfonyldiethanol (2.46%). The variation in the concentration of these compounds from previously reported other extracts of this plant could be due to the seasonal variation, different geographical locations, variation in the extraction procedures, and choice of solvent. The presence of these versatile compounds could be responsible for the strong α-glucosidase inhibition and anti-obesity characteristics of ethanol extract of *M. charantia* fruits.

5. Conclusions

In a nutshell, the phytochemical screening suggested that ethanolic extract of *M. charantia* fruits contained substantial amount of secondary

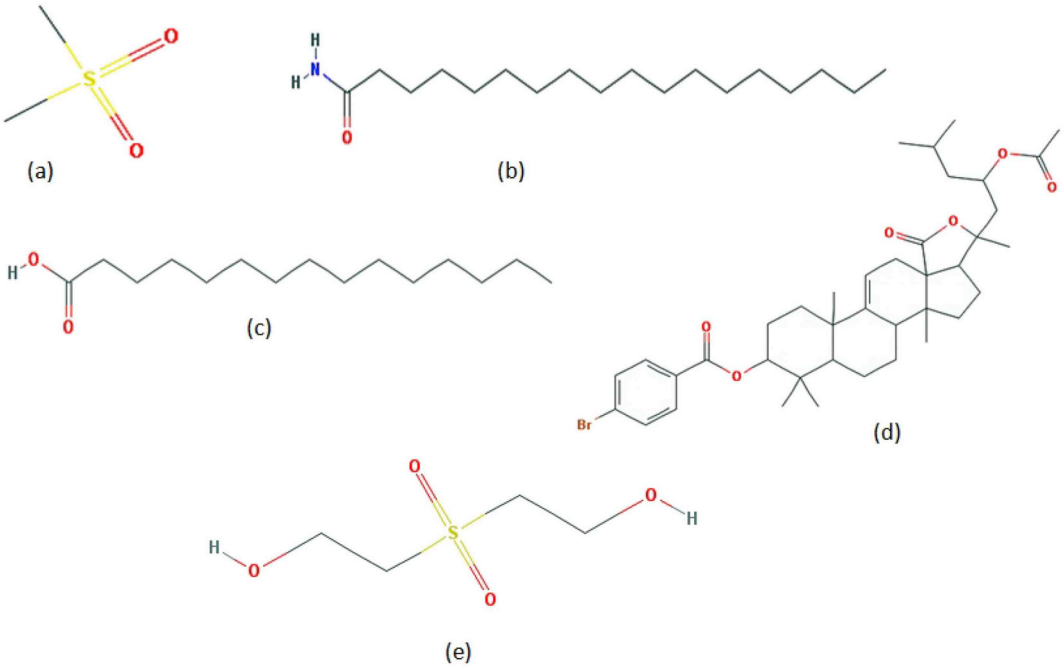

Fig. 3. Major bioactive compounds present in the ethanolic extract of *M. charantia* fruits (a) Dimethyl sulfone, (b) 9-octadecanamide, (c) Pentadecanoic acid, (d) Lanost-9 (11)-en-18-oic acid, 23-(acetylxyl)-3-(4-bromobenzoyl)oxyl-20-hydroxyl-gamma-lactone, and (e) 2,2-sulfonyldiethanol.

metabolites viz. tannins, saponins, quinones, flavanoids, alkaloids, glycosides, cardiac-glycoside, terpenoids, phenols, steroids, coumarins, and betacyanin. The ethanolic extract of *M. charantia* fruit exhibited pronounced activity against poultry associated bacterial pathogens, thereby revealing its potentiality as therapeutic agents for the welfare of public health threatened through poultry sources. Further, the ethanolic extract showed promising inhibition of α-glucosidase in a concentration dependent manner, thereby establishing the scientific foundation for the utility of *M. charantia* fruits in the treatment of diabetes. Additionally, the extract seems to exert beneficial impact on human health by targeting pancreatic lipase, and thus exhibiting the tremendously valuable role of *M. charantia* fruits as anti-obesity agents. The analytical study revealed the presence of several bioactive components in the ethanolic extract of *M. charantia* fruits, predominantly Dimethyl sulfone, 9-octadecanamide, Pentadecanoic acid, Lanost-9 (11)-en-18-oic acid, 23-(acetylxyl)-3-(4-bromobenzoyl)oxyl-20-hydroxyl-gamma-lactone and 2,2 sulfonyldiethanol. The finding demonstrates a great role of *M. charantia* fruits in the healthcare and suggests carrying out further *in vivo* studies in order to combat poultry associated pathogens and epidemic life-style diseases.

Conflicts of interest

None declared.

References

[1] A. Khusro, C. Aarti, J.P. Preetamraj, S.G. Panicker, Comparative study on the effect of different solvent extracts of *Calotropis gigantea* and *Carica papaya* latex against new bacterial isolates – an *in vitro* study, Int. J. Pharm. Pharm. Sci. 6 (2014) 874–879.

[2] Y. Wu, Y. Ding, Y. Tanaka, W. Zhang, Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention, Int. J. Med. Sci. 11 (2014) 1185–1200.

[3] K.H. Mikkelsen, F.K. Knop, M. Frost, J. Hallas, A. Pottegård, Use of antibiotics and risk of Type 2 diabetes: a population-based case-control study, J. Clin. Endocrinol. Metab. 100 (2015) 3633–3640.

[4] T. Kelly, W. Yang, C.-S. Chen, K. Reynolds, J. He, Global burden of obesity in 2005 and projections to 2030, Int. J. Obes. 32 (2008) 1431–1437.

[5] D.P. Guh, W. Zhang, N. Bansback, Z. Amarsi, C.L. Birmingham, A.H. Anis, The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis, BMC Public Health 9 (2009), https://doi.org/10.1186/1471-2458-9-88.

[6] A. Khusro, C. Aarti, J.P. Preetamraj, S.G. Panicker, Antibacterial activity of different solvent extracts of garlic against new strains of pathogenic bacteria: an *in vitro* study, Int. J. Appl. Biol. Pharm. Technol. 4 (2013) 316–321.

[7] A. Khusro, C. Aarti, J.P. Preetamraj, S.G. Panicker, *In vitro* studies on antibacterial activity of aqueous extracts of spices and vegetables against *Bacillus licheniformis* strain 018 and *Bacillus tequilensis* strain ARMATI, Int. J. Curr. Microbiol. App. Sci. 2 (2013) 79–88.

[8] L. Leung, R. Birtwhistle, J. Kotecha, S. Hannah, S. Cuthbertson, Anti-diabetic and hypoglycaemic effects of *Momordica charantia* (bitter melon): a mini review, Br. J. Nutr. 102 (2009) 1703–1708.

[9] B. Joseph, D. Jini, Antidiabetic effects of *Momordica charantia* (bitter melon) and its medicinal potency, Asian Pac. J. Trop. Dis. 3 (2013) 93–102.

[10] Q. Chen, E.T.S. Li, Reduced adiposity in bitter melon (*Momordica charantia*) fed rats is associated with lower tissue triglyceride and higher plasma catecholamines, Br. J. Nutr. 93 (2005) 747–754.

[11] F. Bano, N. Akthar, H. Naz, Effect of the aqueous extracts of *Momordica charantia* on body weight of rats, J. Basic Appl. Sci. 7 (2011) 1–5.

[12] P.H. Chen, G.C. Chen, M.F. Yang, C.H. Hsieh, S.H. Chuang, H.L. Yang, et al., Bitter melon seed oilattenuated body fat accumulation in diet-induced obese mice is associated with cAMP-dependent protein kinase activation and cell death in white adipose tissue, J. Nutr. 142 (2012) 1197–1204.

[13] H.L. Huang, Y.W. Hong, Y.H. Wong, Y.N. Chen, J.H. Chyuan, C.J. Huang, et al., Bitter melon (*Momordica charantia* L.) inhibits adipocyte hypertrophy and down regulates lipogenic gene expression in adipose tissue of diet induced obese rats, Br. J. Nutr. 99 (2008) 230–239.

[14] C.C. Shih, C.H. Lin, W.L. Lin, Effects of *Momordica charantia* on insulin resistance and visceral obesity in mice on high-fat diet, Diabetes Res. Clin. Pract. 81 (2008) 134–143.

[15] L.L. Chan, Q. Chen, A.G. Go, E.K. Lam, E.T. Li, Reduced adiposity in bitter melon (*Momordica charantia*)-fed rats is associated with increased lipid oxidative enzyme activities and uncoupling protein expression, J. Nutr. 135 (2005) 2517–2523.

[16] A. Prakash, T.B. Ng, W.W. Tso, Purification and characterization of charantin, a napin like ribosome-inactivating peptide from bitter gourd (*Momordica charantia*) seeds, J. Pept. Res. 59 (2002) 197–202.

[17] L. Taylor, Technical data report for bitter melon (*Momordica charantia)*, Herbal Secrets of the Rainforest 2nd Ed, Sage Press Inc, 2002.

[18] J.B. Harborne, Phytochemistry, Academic Press, London, 1993, pp. 89–131.

[19] A.W. Bauer, W.M.M. Kirby, J.C. Sherris, M. Turck, Antibiotic susceptibility testing by a standardized single disk method, Am. J. Clin. Pathol. 45 (1966) 493–496.

[20] A. Gutiérrez-Morales, V. Velázquez-Ordonez, A. Khusro, A.Z.M. Salem, M.E. Estrada-Zúñiga, M.Z.M. Salem, et al., Anti-staphylococcal properties of *Eichhornia crassipes, Pistacia vera*, and *Ziziphus amole* leaf extracts: isolates from cattle and rabbits, Microb. Pathog. 113 (2017) 181–189.

[21] C.D. Zheng, Y.Q. Duan, J.M. Gao, Z.G. Ruan, Screening of anti-lipase properties of 37 traditional Chinese medicinal herbs, J. Chin. Med. Assoc. 73 (2010) 319–324.

[22] R. Singh, A. Kumar, D.D. Giri, K. Bhuvaneshwari, K.D. Pandey, Gas Chromatogrphy-Mass Spectrometry analysis and phytochemical screening of methanolic fruit extract of *Momordica charantia*, J. Rec. Adv. Agri. 1 (2012) 122–127.

[23] M. Ullah, M. Showkat, N.U. Ahmed, S. Islam, N. Absar, Evaluation of *Momordica charantia* L. fruit extract for analgesic and anti-inflammatory activities using *in vivo* assay, Res. J. Med. Plant 6 (2012) 236–244.

[24] I.A. Olagunju, B.S. Fagbohunka, O.O. Oyedapo, A.I.A. Abdul, Effects of an ethanolic root extracts of *Plumbago zeylanica* Linn. On some serum parameters of the rats, RPMP- Drug Dev. Mol. 11 (2006) 268–276.

[25] K. Dhama, S.K. Latheef, S. Mani, H.A. Samad, K. Karthik, R. Tiwari, et al., Multiple beneficial applications and modes of action of herbs in poultry health and production-a review, Int. J. Pharmacol. 11 (2015) 152–176.

[26] T.B. Ng, C.M. Wong, W.W. Li, H.W. Yeun, Isolation and characterization of a galactose binding lectin with insulinomimetic activities from the seeds of the bitter gourd. *Momordica charantia* (family Cucurbitaceae), Int. J. Pept. Protein Res. 28 (1986) 163–172.

[27] B.A. Shibib, L.A. Khan, R. Rahman, Hypoglycaemic activity of *Coccinia indica* and *Momordica charantia* in diabetic rats: depression of the hepatic gluconeogenic enzymes glucose-6-phosphatase and fructose-1, 6-bisphosphatase and elevation of both liver and red-cell shunt enzyme glucose- 6-phosphate dehydrogenase, Biochem. J. 292 (1993) 267–270.

[28] L.H. Bosenberg, D.G. Van Zyl, The mechanism of action of oral antidiabetic drugs: a review of recent literature, J. Endocrinol. Metabol. Diabetes S. Afr. 13 (2008) 80–88.

[29] S. Sugiwati, S. Setiasi, E. Afifah, Antihyperglycemic activity of the mahkota dewa [*Phaleria macrocarpa* (scheff.) boerl.] leaf extracts as an alpha-glucosidase inhibitor, Makara J. Health Res. 13 (2009) 74–78.

[30] E. Cummings, H.S. Hundal, H. Wackerhage, M. Hope, M. Belle, E. Adeghate, et al., *Momordica charantia* fruit juice stimulates glucose and amino acid uptakes in L6 myotubes, Mol. Cell. Biochem. 261 (2004) 99–104.

[31] N. Akhtar, B.A. Khan, A. Majid, S. Khan, T. Mahmood, Gulfishan, et al., Pharmaceutical and biopharmaceutical evaluation of extracts from different plant parts of indigenous origin for their hypo hypoglycemic responses in rabbits, Acta Pol. Pharm. 68 (2011) 919–925.

[32] P.V. Nerurkar, Y.K. Lee, V.R. Nerurkar, *Momordica charantia* (bitter melon) inhibits primary human adipocyte differentiation by modulating adipogenic genes, BMC Complement Altern. Med. 10 (2010) 34.

[33] V. Gadang, W. Gilbert, N. Hettiararchchy, R. Horax, L. Katwa, L. Devareddy, Dietary bitter melon seed increases peroxisome proliferator-activated receptor-γ gene expression in adipose tissue, down-regulates the nuclear factor-κB expression, and alleviates the symptoms associated with metabolic syndrome, J. Med. Food 14 (2011) 86–93.

[34] G. Jadeja, R. Katwala, Effect of anti diabetic powder (Fenugreek leaves + Bitter gourd) and green tea on blood sugar status of diabetic patient, J. Cell Tissue Res. 31 (2012) 3125–3131.

[35] S.J. Koona, S. Kudipudi, G.R. Sridhar, S.B. Rao, A. Apparao, Plant insulin: an *in silico* approach, Int. J. Diabetes Dev. Ctries. 30 (2010) 191–193.

[36] M.B. Krawinkel, G.B. Keding, Bitter Gourd (*Momordica charantia*): a dietary approach to hyperglycemia, Nutr. Rev. 64 (2006) 331–337.

[37] Q. Chen, E.T.S. Li, Reduced adiposity in bitter melon (*Momordica charantia*) fed rats is associated with lower tissue triglyceride and higher plasma catecholamines, Br. J. Nutr. 93 (2005) 747–754.

[38] C.C. Shih, C.H. Lin, W.L. Lin, Effects of *Momordica charantia* on insulin resistance and visceral obesity in mice on high-fat diet, Diabetes Res. Clin. Pract. 81 (2008) 134–143.

[39] N.G. Sahib, A.A. Hamid, D. Kitts, M. Purnama, N. Saari, F. Abas, The effects of *Morinda citrifolia, Momordica charantia* and *Centella asiatica* extracts on lipoprotein lipase and 3t3-l1 preadipocytes, J. Food Biochem. 35 (2011) 1186–1205.

[40] A. Ardevol, C. Blade, M.J. Salvado, L. Arola, Changes in lipolysis and sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes, Int. J. Obes. 24 (2000) 319.

[41] S. Adisakwattana, J. Intrawangso, A. Hemrid, B. Chanathong, K. Mäkynen, Extracts of edible plants inhibit pancreatic lipase, cholesterol esterase and cholesterol micellization, and bind bile acids, Food Technol. Biotechnol. 50 (2012) 11–16.

[42] Y.S. Kim, Y. Lee, J. Kim, E. Sohn, C.S. Kim, Y.M. Lee, et al., Inhibitory activities of *Cudrania tricuspidata* leaves on pancreatic lipase *in vitro* and lipolysis *in vivo*, Evid. Based Complement Altern. Med. 2012 (2012) 878365.

[43] H.Y. Lai, S.L. Ong, N.K. Rao, *In vitro* lipase inhibitory effect of thirty two selected plants in Malaysia, Asian J. Pharmaceut. Clin. Res. 7 (2014) 19–24.