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ORIGINAL ARTICLE **OPEN ACCESS**

Phytochemical, Heavy Metal and Metabolomic Anti-diabetic analysis of *Citrus sinensis*

***Syed Rizwan Ahamad**
*Central Laboratory, Research Center, College of pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia
Email – srahamad@ksu.edu.sa, rizwanhamdard@gmail.com

ABSTRACT
Diabetes mellitus is a disease which is increasingly day by day and occurs throughout the world. The rate of increase is very fast in developing countries like Asia and Africa. The urbanization and changes in the lifestyle is completely responsible for the increasing rates of Diabetes. Higher fatty diet alters the metabolism of human body and imbalance of glucose metabolism. A novel approach of metabolomics based on gas chromatography mass spectroscopy was established in our study to explain the Anti diabetic activity of Citrus sinensis. Potential biomarkers were analysed in plasma and they were considered as responsible for metabolic disorder for diabetes. All twenty biomarkers were involved in amino acid metabolism, energy metabolism and lipid metabolism. Therefore Biomarker studies for diabetes associated with GC-MS is a valuable tool to find out the mechanism of Citrus sinensis as anti diabetic agent.
Key Word- *Diabetes, Metabolomics, Citrus sinensis*

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INTRODUCTION

Diabetes mellitus is a chronic disease which effects the whole system of the body. Our body produce hormone insulin which is released by the human body and it can convert extra glucose into glycogen that is stored in the body. Diabetes mellitus is a disease in which our body make enough insulin or it can't use the insulin it does produce, or a combination of both.

High levels of glucose in blood could damage the vital organs of our body like kidneys, heart, eyes, or nervous system. If Diabetes left untreated it can eventually cause heart disease, diabetic nephropathy, diabetic neuropathy and nerve damage. By the end of 2025 the diabetic population may cross 380 million worldwide.[1]. There are different categories of drugs available in the market like insulin, sulfonlyureas, biguanides and glinides. Different class of drugs are available in the market and these drugs showed different side effects like drug-resistance, dropsy, and weight gain [2,3]. So it is a better way to search a hypoglycemic agent from the natural plants that is much more effective and lesser side effects

Antihyperglycemic activity of most medicinal plants performed different types of mechanism to reduce the sugar level in blood by restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or facilitating metabolites in insulin dependent processes [4,5].

Sweet orange belongs to the Family Rutacae. It is grown in tropical and sub-tropical countries. Orange peel is the excellent source of Vitamin C and some essential components present in less amounts. Orange peel contain a lot of phytochemicals like carotenoids ,flavonoids, lutein and beta carotene.

Citrus sinensis showed a lot of pharmacological activities like antioxidant, antidiabetic (6), antipeptic ulcer potential [7]. anti-inflammatory and pain reducing activity in rats (8) still there is no metabolomics approach to describe the antidiabetic activity.

Metabolomics have been studied thoroughly by various methods using NMR, LC-MS and GC-MS. It has numerous advantages in comparison to traditional method is that of higher sensitivity, better availability and easier identification of the metabolites with the help of libraries.

In this work the anti-diabetic activity of *Citrus sinensis* are studied by metabolomics using GC-MS and evaluate various levels of amino acids, fatty acids and sugars. By analyzing all the potential biomarkers concentration we can access the possibility and cure by *Citrus sinensis*

MATERIAL AND METHODS

PHYTOCHEMICAL ANALYSIS BY GC-MS

Chemicals-

Methoxylamine hydrochloride, bis-N-(trimethylsilyl) trifluoroacetamie (BSTFA), Trimethylchlorosilane (TMCS) and undecane (used as internal standard) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Deionized water was purified by the Milli-Q system (Millipore, Bedford, MA, USA). Allthe reagents were chromatographic grade.

Plant Material and Preparation of Extract

The fruits of *Citrus sinensis* were purchased from the local market in Riyadh. The peel of fruit clearly separated with the help of sharp knife and cut into small pieces and dried in the drying room. The dried peel (500 g) was coarsely powdered and macerated in 5 litres of ethanol for 72 h using the percolation method. The solvent was then removed at 40 °C under reduced pressure in a rotavapor. For GC-MS analysis, a 200-μL sample of ethanolic extract was placed in a GC vial and evaporated under nitrogen stream. In this vial 100 μL ofmethoxamine in pyridine (20 mg/mL) was added. After overnight incubation at room temperature, 100 μLN, O-bis-trimethyl tri-fluoroacetamide (BSTFA) was added along with 50 μL of hexane. This final mixture was subjected to analysis by GC-MS.The GC-MS analysis was accomplished in a gas chromatograph (Perkin-Elmer, Autosystem XL)linked to a mass spectrometer (Turbomass) available at the Research Center, College of Pharmacy King Saud University. An aliquot of 1μL of extract was injected into the Elite-5MS column of 30m× 0.25mm internal diameter of 0.25-μmfilm thickness glass capillary column using the following temperature program: initial oven temperature of 40°C for 2 minutes, increasing to 300°C at a rate of 5°C/min with a hold for 2 minutes at the ending temperature. The injector temperature was maintained at 250°C. The interface temperature was 200°C and the GC line temperature is at 220°C; the electron energy used was 70 eV. The scan duration was 1.0 second and the inter scan delay was 0.4 seconds. Helium was used as a mobile phase at a flow rate of 1.0 mL/min. Mass spectral detection was carried out in electron ionization mode by scanning at 40-550 (m/z). Finally, unknown compounds in Table-3 were identified by comparing the spectra in with that of the National Institute of Standard and Technology(NIST) 2005 Library and Wiley AccessPak v7 May,2003 library. The total time required for analyzing a single sample was 58 minutes.

TRACE ELEMENT DETECTION BY ICP-MS

Instrumentation

An inductively coupled plasma mass spectrometer, PerkinElmer SCIEX, ELAN DRC-e (Thornhill, Canada) was used. The argon gas with minimum purity of 99.996% was supplied by Air Liquidi. An HF-resistant and high performance Teflon Nebulizer model PFA-ST, was coupled to a quartz cyclonic spray chamber with internal baffle and drain line cooled with the PC3 system from ESI (Omaha, NE, USA) (Table 1). Tygon black/black 0.76 mm i.d. and 40 cm length peristaltic pump tubing was used. The instrument conditions were: auto lens mode on, peak hopping measure mode, dwell time of 50 ms, 15 sweeps/reading, 1 reading/replicate, and 3 replicates. Nickel sampler and skimmer cones were used. Before changing to the microconcentric nebulizer, a performance check for sensitivity and oxide and doubly charged ion formation, using a conventional cross flow nebulizer and a Scott spray chamber was carried out. The ICP-MS operational conditions are summarized in Table 1.

Table 1. Instrumental conditions of the ICP-MS.

S. No.	**ELAN 9000 Parameters**	**Value**
1	RF Power	1500 watts
2	Plasma Gas Flow	15 L/min
3	Auxiliary Gas Flow	1 L/min
4	Nebulizer Gas Flow	0.83-0.88 L/min
5	Peristaltic Pump Speed	0.5 mL/min
6	Nebulizer/Spray Chamber	PFA-ST/Peltier-cooled cyclonic
7	Spray Chamber Temp	2 °C
8	Detector Mode	Dual Lens / AutoLens Enabled
9	Sampler/Skimmer	Cones Nickel
10	Scanning Mode	Peak Hopping
11	Number of Points/Peak	1
12	Number of Sweeps/Reading	10
13	Number of Replicates	3

Tuning and Internal Standards

1 ppb solution containing Be, In, se, U, Rh, Mg, Co, Pb, Na, Fe, Cu, Pb, Ba in 1% HNO_3 was used for all instrument tuning and optimization. This tuning solution was used to measure all performance aspects of the instrument, including mass calibration, resolution, nebulizer gas flow, Auto Lens calibration, and daily performance checks. The tuning solution was prepared by diluting 50 µL of a 10 ppm multi-element stock solution into 500 mL of 1% HNO_3. The multi-element stock solution is prepared from 1000 ppm single element stock solutions of the elements listed above by diluting 500 µL of each element into 50 mL of 1% HNO_3.

ANTI DIABETIC ACTIVITY

Animals

Male Wistar albino rats, 12 weeks of age, weighing 160–180 g collected from the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia were used in the experiments. The animals were kept at a constant temperature (24 ± 2 °C), humidity (55±5%) and light-dark conditions. The animals were provided with normal diet and drinking water.The procedure of experiments and sacrifice (using ether) were approved by the Ethics Committee of the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Overnight-fasted animals were randomly divided into three groups of six rats:

Diabetes was induced by a single intraperitoneal injection of 200 mg/kg of alloxan monohydrate (dissolved in water just before use). Animals in which the development of hyperglycemia was confirmed (around 60%), 72 h afterthe alloxan injection, with serum glucose levels >300 mg/dl were considered diabetic. These were used for the studyand they were randomly allocated into groups of six rats:

Group I: control and received the vehicle only

Group II: *Citrus sinensis* ethanolic extract 250 mg/kg

Group III: glibenclamide 200 mg/kg

Blood samples were collected after 2 hours of administration of *Citrus sinensis* extract.

Sample preparation-ll mice were sacrificed after treatment of 72 h of being induced with alloxan. Blood were collected into heparinized tubes, and then centrifuged at 10000 rpm and 4°C for 10 min. The plasma was collected and was frozen at -80°C until GC-MS analysis. Animal welfare and experimental procedures were always per-formed according to the protocol approved by the Animal Ethics Committee of King Saud University.

METABOLOMIC STUDY BY GC–MS

The plasma samples were thawed at room temperature prior to analysis. 250 µL of acetonitrile was added into100 µL of the sample in 1.5 mL centrifuge tube to precipitate theprotein. The mixture was ultrasonically extracted for 10 min, followed by centrifugation (10000 rpm) for another 10 min. 400 µL of supernatant was then transferred to the centrifuge tube and evaporated to dryness under a stream of nitrogen gas. Approximately100 µL of methoxyamine HCl/pyridine solutions (20 mg/mL) was then added to the vial. Methoxymation was performed at 60°C for 1 hr,and then 50 µL of BSTFA with 1% TMCS was added to the vial. After the silylation was performed at 70°C for 1 h, 150 µL of n-heptane. The mixture was then centrifuged for 10 min, andthe supernatant was transferred to the GC microvial for GC–MS analysis.

Chromatography

GC–MS was applied to analyze the plasma samples. Chromatographic analysis was performed in an Perkin Elmer Clarus 600T series GC–MS equipped with an Elite 5MS capillary column (30 m × 0.25 µm × 25 mm).The initial temperature (40◦C) was held for 5 min and then raised to 150°C at a rate of 5°C/min and again increases to 300°C and hold upto 5 min. All samples were injected in split mode. The injection temperature was 200°C. The mass spectrometer was operated in EI mode (positive ion, 70 eV), and the quadrupole was 220°C. Mass spectra was acquired in full scan mode with repetitive scanning from 40 m/z to 600 m/z in 1 s. Ion source temperature was 230°C.

RESULT AND DISCUSSION

Metabolomics have been used to identify the potential biomarkers in relation with Diabetes. Various biomarkers have been identified by gc-ms based metabolomics and find out the metabolomics pathway of the metabolome. Lactic acid is responsible for carbohydrate and amino acid metabolism if the concentration of the lactic acid increase so there is imbalance in between carbohydrate and amino acid metabolism. Urea and glucose also related to carbohydrate and amino acid metabolism. These finding played a key role in finding the metabolome of diabetes. Liver is related to amino acid metabolism and change in the concentration of Glycine, L-Proline is responsible for the role of diabetes . Most of the amino acids are metabolized in liver hence directly related with the activity of hepatic enzymes. Glycine and

proline are essential amino acids and they are responsible for stress energy and muscle related metabolism.

Long chain fatty acids also responsible for energy management in the body. Tetradecanoic acids, hexanedecanoic, octadecanoic acids and palmitinic acids were significantly increased in diabetic patients and it is decreased in the treated group of animals.

The heavy metal analysis showed a high concentration of Magnesium, Phosphorus, Manganese , Sulphur, iron , potassium and calcium. Magnesium ions is present in nucleic acids and synthesis of DNA and RNA. Phosphorus is responsible for energy and key component of ATP and responsible for neurological functioning of brain. Manganese is responsible for mental health and responsible for development, metabolism and antioxidant system. Manganese is a component of hydrolases, oxidoreductases and polypeptides. Sulphur is a important component of amino acids like cysteine, taurine, homocystiene and methionine and also present in polypeptide, proteins and enzymes. Iron is key component of haemoglobin so it is responsible for the oxygen carrier in the body. Potassium and calcium played a key role in neurological transmission and bones respectively.

Biomarkers	**Control**	**Disease**	**Treated**
Lactic acid	563930	2429787	905632
Urea	47375	1447042	1125632
Glycine	7662	37179	130564
L-proline	47331	96734	44077
Tetradecanoic acid	40006	99724	190364
D glucitol	1168359	27130914	8563225
Hexadecanoic acid	130791	911377	563256
Oleic acid	14128	1319069	413260
Octadecanoic acid	247000	3165815	863146
Palmitinic acid	60539	367034	287017
Beta-D glycopyranoside	25044	68285	11675

Biomarkers	**change**	**source of pathway**
Lactic acid	↑	carbohydrate and amino acid metabolism
Urea	↑	carbohydrate and amino acid metabolism
Glycine	↑	abnormal metabolism of amino acid
Lproline	↑	abnormal metabolism of amino acid
Tetradecanoic acid	↑	decreased metabolism of uric acid and tricarboxylic acid cycle
Hexadecanoic acid	↑	decreased metabolism of uric acid and tricarboxylic acid cycle
Oleic acid	↓	Change of serum phospholipid content
Octadecanoic acid	↑	`decreased metabolism of uric acid and tricarboxylic acid cycle
Palmitinic acid	↑	decreased metabolism of uric acid and tricarboxylic acid cycle
Beta-D glycopyranoside	↓	prevented AGE-mediated decrease in PPARγ activity

Trace elements estimated by ICP-MS

Table 2. Concentration of different elements in µg/g (ppm) of *Citrus sinensis* by ICP-MS.

S. No.	**Elements**	**Symbol**	**Molecular weight**	**Concentration (µg/g)**
1	Lithium	Li	6.941	1.91
2	Beryllium	Be	9.012182	0.01
3	Boron	B	10.811	125.6
4	Sodium	Na	22.989769	138.2
5	Magnesium	Mg	24.305	28.3
6	Silicon	Si	28.0855	195.3
7	Phosphorous	P	30.973762	130.9
8	Sulphur	S	32.065	2184.9
9	Chlorine	Cl	35.453	260.3

10	Potassium	K	39.0983	829.3
11	Calcium	Ca	40.078	91.2
12	Scandium	Sc	44.95591	0.02
13	Titanium	Ti	47.867	0.11
14	Manganese	Mn	54.938045	0.247
15	Iron	Fe	55.845	25.34
16	Nickel	Ni	58.6934	1.0801
17	Zinc	Zn	65.38	0.5795
18	Iodine	I	26.90447	0.5833

Table-3 Phytochemicals identified in *Citrus sinensis*

	Name	RT	Area	N Area %	Area %
1	Arabinitol	5.52	36280	2.770	0.340
2	Octadecanol	6.01	10254	0.780	0.100
3	Di-(9-Octadecanoyl)-glycerol	8.81	36995	2.820	0.350
4	Ethyl Propyl Maleate	18.99	206361	5.750	1.940
5	Gamma Octadeca lactone	21.78	54686	4.180	0.510
6	3-Hydroxy tetradecanedioc acid	24.47	435541	33.250	4.090
7	Tetracosanoic acid	26.03	264721	20.210	2.480
8	1,4-Heptanediol	27.06	84186	6.430	0.790
9	TetrahydroLinalyl acetate	29.58	86615	6.610	0.810
10	6-Methyl-2,5-Heptadecadiol	30.70	185825	4.190	1.740
11	Propanoic acid	31.54	375352	8.660	3.520
12	D-Arabinohexonic acid	31.89	441289	33.690	4.140
13	alpha dl arabinopyranoside	32.22	370379	28.280	3.480
14	Prost-13-en-1-oic acid	32.71	121263	9.260	1.140
15	2,5-Octadecanol	33.41	139164	10.620	1.310
16	D-Ribopyranose	33.52	407972	31.150	3.830
17	D-Glyceraldehyde	33.83	598633	5.700	5.620
18	Gluconic acid	34.06	1309817	100.000	12.290
19	1-O-D-Mannitol-Docosane	34.48	22750	1.740	0.210
20	2-ethylhydrallic acid	34.60	52680	4.020	0.490
21	Erythretol	34.88	666474	50.880	6.260
22	D-Ribose	35.44	41141	3.140	0.390
23	1-Hexadecanol	35.97	110968	8.470	1.040
24	Hexadecanoiccaid	36.65	914622	69.830	8.580
25	Ethyl lineolate	39.66	363824	27.780	3.410
26	3-methyl-7-carboxyldehyde	39.78	230391	17.590	2.160
27	9,12-Octadecanoic acid	40.50	41772	3.190	0.390
28	11-trans octadecanoic acid	40.59	45157	3.450	0.420
29	Octadecanoic acid	41.08	55702	4.250	0.520

CONCLUSION

A novel approach using biochemical parameters related to metabolomics based on GC-MS was established and to explain the anti diabetic effect mechanism. Long chain fatty acids , amino acids and sugar biomarkers in plasma were found. *Citrus sinensis* markedly decrease the level of long chain fatty acids, amino acids and glucose level in treated groups.

DECLARATION OF INTEREST

The author report no conflict of interest. The author alone are responsible for content and writing of paper.

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