GC-MS Analysis of Chemical Constituents of Hydroalcoholic Leaf Extract of Cissampelos Pareira and Their Anti-Diabetic Activity

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Authors’ contributions

This work was carried out in collaboration among all authors. Author MA designed the study and conducted the experiment. Author SJG contributed in literature survey and wrote the protocol. Author MT performed statistical analysis. Author CK drafted the manuscript and involved in correspondence. Author DG provided the permission to conduct the experiment in the place of work. Author IR managed the literature searches. Author NAK supervised the work. All the authors read and approved the final manuscript.

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ABSTRACT

Aim: The present work deals with the GC-MS-analysis of chemical constituents of hydroalcoholic extract of Cissampelos pareira leaves and their anti-diabetic activity.

Methods: GC-MS analysis of extract was performed using Shimadzu QP-2010 plus with thermal desorption system 20. Acute oral toxicity of extract was done using the Organization of Economic Co-operation and Development (OECD) guideline 423. Diabetes was induced by single dose of streptozotocin 65 mg/kg, i.p. to all the rats except in rats of control group. Following which oral glucose tolerance test was performed and the rats were divided into various experimental groups. Various treatments continued for 21 days. Parameters such as blood glucose level, body weight, liver enzymes, lipid profiles and oxidative markers were checked.

Results: GC-MS analysis of the extract identified 25 compounds present in it. Based on acute oral toxicity study three doses of hydroalcoholic extract of Cissampelos pareira leaves viz 100, 200 and 400 mg/kg were selected for evaluation of anti-diabetic activity. The extracts at doses 200 and 400 mg/kg BW were able to reduce blood sugar level, liver enzymes, total cholesterol, total triglyceride, low density lipoprotein and Malondialdehyde; and enhance body weight, high density lipoprotein and Glutathione significantly when compared to rats of negative control group. The effect of extract at dose 400 mg/kg was comparable to standard Glibenclamide.

Conclusion: Results conclude that the chemical constituents present in the hydroalcoholic extract of Cissampelos pareira contained some anti-diabetic compounds possessing strong anti-diabetic activity.

Keywords: Cissampelos pareira; GC-MS analysis; streptozotocin; glibenclamide; blood glucose level; liver enzymes; lipid profile; oxidative stress markers.

1. INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by chronically increased blood glucose levels attributed to an abnormality in secretion and/or action of insulin. Low level of insulin or insulin resistance is responsible for metabolic abnormalities in carbohydrates, lipids, and proteins. The severity of symptoms is dependent upon type and duration of diabetes. Marked hyperglycemia with absolute insulin deficiency may result in symptoms like polyuria, polydipsia, polyphagia, weight loss and blurred vision. Uncontrolled diabetes may result in ketoacidosis or nonketotic hyperosmolar syndrome (rare) leading to stupor, coma and if untreated, death [1].

Mainstream drugs for diabetes includes sulfonylureas, thiazolidines, meglinides, d-phenylalanine, α-glucosidase inhibitors, insulin treatment with modification in diet and lifestyle. However, such treatment cannot be termed as an ideal one owing to its toxic side effect and reduced response over a period of time. As a result, the search for newer drug especially those of natural origin has dragged massive attention of researchers [2].

In last few decades the incidences and complications of diabetes have been increased substantially owing to changes in lifestyle and living condition. As per one of the report, in 2017, approximately 451 million adults (8.4%) were diagnosed with diabetes across the globe and these figures are estimated to reach around 693 million by 2045 [3].

Traditional plant-based medicine has been extensively used and of grave importance all over the globe. The World Health Organisation (WHO) has enlisted 21000 plants of medicinal importance and more than 400 plants for diabetes treatment. The presence of phytoconstituents such as terpenoids, flavonoids and coumarins are counted for the anti-diabetic nature of the medicinal plants. Picnagenol, acarbose, miglitol, and voglibose are some of the marketed anti-diabetic drugs of natural origin [4].

Cissampelos pareira (C.pareira) is a climbing herb distributed in tropical and subtropical war regions of Africa, Asia, and America [5].

C.pareira has many pharmacological effects. According to Ayurvedic Pharmacopeia of India, C.pareira is ascribed for treatment of abdominal pain, asthma, arthritic, cough, diarrhea, fever, heart disease, kidney stone and kidney infections. In South America, it is used by natives for treating menstrual cramps, preventing miscarriage, easing childbirth, ceasing uterine hemorrhages after childbirth attributed to its
property for relaxing smooth muscle. Leaves of C. pareira are traditionally used in the treatment of ulcers, urinary tract infections [6,7].

C. pareira contains various pharmacologically active phytoconstituents. The whole plant is the rich source of alkaloids. For instance, leaves are rich in Laudanosine, dicine, magnoflorine, nuciferine; roots are rich in insularine, cycleanine, cissampareine, (-) - curine, hayatinine, isochondodendrine, pelosine, (+)berberine, diajisong, (+)-cortyuberine, dihydrodencitrine, steponine, (-)-cissamine, (-)-β-cyclanolone, isoimerubrine, pareirubrine A & B, grandirubrine, pareitropone, norimeluteine, norruffscine, seepereine, (-)-oblongine, (+)-obaberine, (+)-obamegine, (+)- homoaromoline, (-)-nor-N’-chondrocurine, (+)-tetrandrine, berberine, phanostenine; stems contain cissampareine, magnoflorine, nuciferine. The leaves are also rich in flavonoids viz quercetin 3-O-sophoroside, naringenin 7-OD-glucoside, eriodictyl-7-O-beta-D-glucoside, galangin-7-glucoside and baicalein-7-O-glucoside; roots have been reported to have quercetin and its aerial part is known to contain cissampeloloflavoid [6,7].

The present study has been undertaken to identify chemial constituents present in hydroalcoholic extract of C. pareira using GC-MS analysis and to evaluate their anti-diabetic potential.

2. MATERIALS AND METHODS

2.1 Plant Material

Fresh leaves of C. pareira were identified and purchased from creative farmer live plant site, online. The plant species was authenticated from Botanical Survey of India (BSI), Jodhpur Rajasthan (Voucher no, BSI/AZRC/I.12012/Tech./2019 (Pl. ID.) 549).

2.2 Drug And Chemicals

Streptozotocin was purchased from Hi-media lab pvt. Ltd. Mumbai, India. Glibenclamide (5 mg) tablet was purchased from Sri Pharmacare, Mumbai. Kit for liver function test was purchased from Avecon healthcare pvt ltd, Ambala, Haryana, India. Kit for determination of lipid profile was purchased from Tulip diagnostic pvt. Ltd. All the chemicals and reagent used were laboratory reagent (LR) grade and were purchased from LobaChemie Ltd., Mumbai.

2.3 Experimental Animals

Swiss mice and wistar albino rats of either sex were purchased and housed in the animal house of Bilwal Medchem and Research Laboratory Pvt. Ltd., Reengus Industrial Area (RIICO), Sikar, (Raj.) under standard environmental conditions of temperature (25 ± 2 °C), relative humidity (45 - 55 %) and 12 h dark/light cycles. All animals were fed with standard diet (standard pellets, Hafed, Rohatak India) and water ad libitum.

2.3.1 Extraction of Leaves of C. pareira

Leaves of C. pareira were cleaned and air dried under shade under room temperature until left with no moisture (15 days). The dried leaves were coarsely powdered in the mixer and stored in an air-tight container until further use. The dried coarse leaves (250 g) were defatted with petroleum ether (6000 ml) using continuous extraction. The extraction was discontinued after 10-12 cycles after complete defatting. The defatted extract was then evaporated at 40 °C in a rotary evaporator and then dried to obtain marc. The dried marc was further extracted with a mixture of ethanol:water (5:5) (1000 ml) using continuous extraction until a drop of solvent from the siphon tube became clear. The obtained extract was filtered, concentrated, and stored until further use [8].

2.4 Gas Chromatography - Mass Spectroscopy Analysis

GC-MS analysis of obtained extract was performed using Shimadzu QP-2010 plus with thermal desprtion system 20. The operating parameters for the same were as follow: Splitless injection mode, temperature-programmed from 50 to 315°C at 5°C/min with a 2-min initial and a 10-min final temperature hold; Helium carrier gas flow rate at 1.1 ml/min, with the injector temperature set at 250°C. Mass spectra were obtained by electron impact ionization (EI) over the range of 40–550 amu at a rate of 2scans/s. The ion source temperature was set at 180°C and the electronic impact energy was maintained at 106eV. The identification of components was based on Willey8 and NIST1 as well as retention indices.

2.5 Acute Oral Toxicity Study

Animals were divided into five groups containing three animals in each group (n=3). The animals
have fasted overnight. They were administered with 5, 50, 300 and 2000 mg/kg B.W of extract. Afterward, the animals were observed initially for 30 minutes, then at 4 hours of administration and then for every 24 hours for 14 days for any sign of toxicity [9].

2.6 Experimental Group

Wistar albino male rats were used for study. The animals were divided into six groups containing six mice (n=6) in each group. The control group received the vehicle, the negative control group received only streptozotocin (5 mg/kg) and the treatment groups received 100, 200, and 400 mg/kg B.W of C.pareira leaves extract. The dose of the test extract was selected based on acute oral toxicity. As no toxicity was observed till 2000 mg/kg B.W., 1/10 of 2000 mg/kg i.e. 200 mg/kg B.W was taken as the middle dose; a double dose of 200 mg/kg B.W i.e. 400 mg/kg B.W was taken as high dose; and half of middle dose i.e. 100 mg/kg was taken as the low dose. Low dose, middle dose and high dose of test extract were designated as CPLD, CPMD and CPHD respectively [10].

2.7 Induction of Diabetes in Experimental Animals

The experimental animals were fasted for 16 h and their basal body weight (g) and fasting blood sugar (mg/dl) were determined. A single dose of streptozotocin (65 mg/kg, i.p) prepared in 0.1 ml of freshly prepared cold sodium-citrate buffer (NaB – 0.1 M, pH 4.5) was used to induce diabetes. Immediately after STZ administration, animals were administered with 5% of glucose solution for 2 days to prevent severe hypoglycaemic shock. Animals in the control group did not receive STZ. Confirmation of diabetes was carried out by measuring the blood glucose level of rats using Accu check active glucometer machine, India, on the 3rd day after STZ administration [11].

2.8 Oral Glucose Tolerance Test (OGTT)

Post 3 days of diabetes induction, the fasting blood sugar of rats was recorded and the rats with the levels ≥ 300 mg/dl were selected. OGTT was conducted to check glucose tolerance capacity and to confirm the development of diabetes [12]. For OGTT, rats were fasted overnight (18 h) and their baseline glucose level was recorded. After 30 mins of their respective treatment, they were administered with Glucose (3 g/kg). Afterward, the blood glucose level of each rat of various group is recorded at 0, 30, 60, 90 and 120 minutes of glucose administration by Accu check active glucometer machine, India [11,13].

Animals with blood sugar level more than 300 mg/dl were randomly distributed in various experimental groups as follows: Group I : Normal control (water ad libitum ); Group II : Diabetic control; Group III: standard (Glibenclamide 0.5 mg/kg); Group IV: Test group (CPLD 100 mg/kg); Group V: Test group (CPLD 200 mg/kg); Group VI: Test group (CPLD 400 mg/kg). The treatment was continued for 21 days and alteration in body weight and blood sugar level were measured at 1, 7, 14, and 21st day.

2.9 Biochemical Estimation

2.9.1 Estimation of liver enzymes and lipid profile

At the end of the 21st day, serum from rats was obtained by centrifuging the blood at 3000 rpm for 15 minutes at 4° C. Serum was collected and stored at 4° C for estimation of HDL, LDL, Cholesterol, Triglycerides, AST, ALT and total bilirubin [13].

2.9.2 Determination of In-vivo anti-oxidant activity (Estimation of GSH and MDA)

Tissue homogenate was prepared by homogenizing 1 g of frozen tissue in 10 ml of 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 10 minutes and the supernatant was collected for determination of GSH and MDA [14]. The concentration of protein was determined using Lowry et al utilizing standard purified bovine serum albumin [15].

2.9.2.1 Estimation of glutathione (GSH) level

The equal quantity of supernatant and Tricholoroacetic acid (10%) was mixed and the content was centrifuged for separation of protein. To the supernatant (0.01 ml) obtained, phosphate buffer (2 ml, pH 7.4), 5.5’ dithiobisnitro benzoic acid (DTNB) (0.5 ml), and double distilled water (0.4 ml) were added. The absorbance of the mixture was taken at 412 nm. GSH was measured as µg/g of protein [16].
2.9.2.2 Determination of lipid peroxidation (measurement of MDA/TBARS) level

1 ml of the supernatant was added to the tube containing 30% trichloroacetic acid (TCA) (0.5 ml) and 8% of thiobarbituric acid (TBA) (0.5 ml) reagent. The tubes were covered with aluminum foil and was placed in a shaking water bath for half an hour. The absorbance was taken at 540 nm. MDA was measured as nm/g of protein [16].

2.10 Statistical Analysis

Values are expressed Mean ± Standard error of Mean (SEM), number of animals per group (n) = 6. Means of all the treatment groups were compared with the mean of negative control group by one way ANOVA followed by Dunnett’s multiple comparison test and the criterion for statistical significance was p<0.0001, p<0.01, p<0.05; and mean of the control group was compared with the mean of the negative control group by unpaired t-test and, the criterion for statistical significance was p<0.001, p<0.01 and p<0.05 using Graph Pad Prism (9.0.0).

3. RESULTS AND DISCUSSION

3.1 GC-MS Analysis

The present study deals with the GC-MS analysis and anti-diabetic potential of hydroalcoholic extract of Cissampelos pareira. GC-MS analysis of a hydroalcoholic extract of leaves Cissampelos pareira was carried out to identify bioactive compounds present in it. GC-MS is the most popular technique contributing to the discovery development and registration of new compounds [17]. Presently, GC-MS is integrated with several online libraries containing reference compounds so that separated components can be identified by matching the available spectra [18]. The mass spectra of the constituents were compared with Wiley and NIST1 library based on which 25 components were characterized. GC-MS analysis was done to identify the bioactive component in Cissampelos pareira hydroalcoholic extract. 25 compounds were separated and identified. The major portion includes Mome-inositol (79.52%). Identified components are presented in Table 1 and Fig. 1.

3.2 Induction of Diabetes

Diabetes into the experimental rats was induced by intraperitoneal injection of Streptozotocin. Introduction of streptozotocin begins an autoimmune process and a dose of more than 40 mg/kg leads to islets of langerhans beta cell destruction with the emergence of clinical diabetes within 2-4 days [19,20]. After the administration of STZ in rats OGTT was carried out and rats with blood glucose levels of more than 300 mg/dl were distributed among various treatment groups. Afterward, a 21 days study was started to evaluate the anti-diabetic property of C. pareira extract.

3.3 Acute Oral Toxicity Study

The acute oral toxicity was done as per OECD 423 guidelines. The animal does not display any sign of toxicity or behavioral changes during the period of two weeks. Based on acute oral toxicity study, three doses viz 100, 200, and 400 mg/kg were selected for further pharmacological evaluation. Since the animal was found to safe and healthy till 2000 mg/kg BW, 1/10th of 2000 mg/kg, i.e. 200 mg/kg was selected as middle dose; half of 200 mg/kg, i.e. 100 mg/kg was selected as lower dose and double of 200 mg/kg i.e. 400 mg/kg was selected as high dose.

3.4 Effect on Body Weight

The body weight and blood glucose level of the experimental rats were checked at 0, 7, 14, and 21 day of study. In our study, negative control group rats showed a decrease in body weight throughout the experimental period. Oral treatment with C. pareira extract for 21 days improved body weight compared to the negative control group. The bodyweight of negative control group rats decreased significantly when measured on day 14 and day 21 (p<0.01 and p<0.0001, respectively) compared to normal control group rats. The weight of normal control group rats increased throughout the study from 220.67 ± 2.9 gm. However significant increase in body weight of the rats was observed when measured at day 21 compared to negative control group at p<0.05. In rats of CPMD and CPHD groups, gradual increase in the body weight was observed compared to the negative control group rats and this effect was highly significant when measured at day 21 (p<0.0001). Results are presented in Table 1.
Fig. 1. GC-MS analysis of hydroalcoholic extract of leaves of *Cissampelos pareira*
often called 'diabetic dyslipidemia' characterized by enhanced total cholesterol, triglyceride, LDL, and low HDL [21]. Therefore, in our study lipid profile of rats of all experimental groups was measured. Rats in the negative control group showed increased TC, TG, and LDL and decreased HDL. Contrarily, treatment with *C. pareira* extract showed potent activity in lowering TC, TG, and LDL and enhancing HDL.

The rats of the negative control group displayed a significant increase (p<0.0001) in total cholesterol (TC), triglyceride (TG) and low-density lipoprotein (LDL) and a significant decrease (p<0.0001) in high-density lipoprotein (HDL) when compared with rats of the normal control group. Administration of CPHD significantly decreased (p<0.0001) TC, TG, and LDL and significantly increased (p<0.0001) HDL in rats compared to the rats of the negative control group. Administration of CPMD significantly decreased (p<0.0001) TC, TG, and LDL, however, an increase in HDL level was not significant. Rats in CPLD group showed a significant decrease in TC and TG (p<0.0001 and p<0.05 respectively). However, decrease in LDL and an increase in HDL were not significant.

### 3.5 Effect on Blood Glucose Level

A significant increase in blood glucose level was observed in rats of the negative control group throughout the study as compared to normal control (p<0.0001). Repeated oral administration of *Cissampelos pareira* for 21 days to diabetic rats produced an anti-diabetic effect when compared to the negative control group. Among all doses, CPHE (400 mg/kg) produced significant a decrease in blood glucose from 338.36 ± 5.35 to 157.61 ± 2.86 mg/dl at p<0.0001 when measured on day 7, 14, and 21, compared to rats of the negative control group. Rats in the CPMD group also showed a significant decrease in blood glucose level from 348.48 ± 6.4 to 223.17 ± 3.33 mg/dl when measured on day 7 (p<0.0001) and at day 14 and 21 (p<0.0001). Contrarily, a decrease in blood glucose level in rats of the CPLD group was not significant. Results are presented in Fig. 3.

### 3.6 Effect on Lipid Profile

Dyslipidemia is a major risk factor for diabetes; lipid abnormalities in patients with diabetes are often called ‘diabetic dyslipidemia’ characterized...
when compared to the negative control group. Results are presented in Fig. 4.

3.7 Effect on Liver Enzymes

The liver plays a pivotal role in glucose homeostasis, as it extracts glucose from blood and use as fuel and carries glycogenesis, gluconeogenesis, and glycogenolysis. Therefore liver function test is a widely requested biochemical test recommended by the clinician to know about the functional state of Diabetic patient's liver. Studies suggest that elevated liver enzymes such as ALT, AST, ALP and gamma-glutamyl transpeptidase (GGTP) in serum in common in a diabetic patients [22]. Therefore, in the present study level of liver enzymes was measured. The negative control group showed elevated liver enzymes. CPMD and CPHD significantly lowered liver enzymes after 21 days of treatment compared to the negative control group. The result reveals that significant increase (p<0.0001) in liver enzymes (AST, ALT and ALP) was observed in negative control group rats compared to the normal control group rats. Treatment with CPMD and CPHD significantly decreased the values of liver enzymes at p<0.0001 when compared to negative control group. Contrarily, the effect of CPLD in lowering ALP was not significant; and in lowering of AST and ALT was significant at p<0.05 and p<0.001 respectively, compared to negative control group rats. Results are presented in Fig. 5.

3.8 Effects on Oxidative Stress Markers

Oxidative stress as a result of hyperglycemia leads to dysfunction and death of beta cells affecting insulin sensitivity, and pancreatic islets fibrosis. Elevated glucose upon autooxidation gives rise to free radicals which cause lipid peroxidation of the cell membrane and protein glycation. Chronic hyperglycemia results in reduced antioxidant enzymes such as glutathione peroxidase (GSH) and superoxide dismutase (SOD); enhanced protein glycosylation and oxidative phosphorylation [2,3]. Therefore the effect of *C.pareira* extract on oxidative stress marker GSH and MDA was checked. The extract significantly increased GSH level and decreased MDA level in both liver and pancreatic tissue which indicate that rats treated with extract have been recovered from the hyperglycemia induced oxidative stress.

![Fig. 2. Effect of various treatments on body weight of experimental rats](image-url)
The result of the study reveals a significant decrease (p<0.0001) in GSH level in liver and pancreas tissues of negative control group rats compared to normal control group rats. Administration of CPLD, CPMD, and CPHD significantly increased GSH level in both the tissues at p<0.001, p<0.0001, and p<0.0001 respectively. Also, a marked increase (p<0.0001) in MDA level was observed in liver and pancreas tissue of negative control group rats compared to normal control group rats. Treatment with CPMD and CPHD displayed a significant decrease in MDA levels in both the tissue at p<0.001 whereas the effect of CPLD in lowering MDA in liver tissue was significant at p<0.01 and non-significant in pancreas tissue. Results are presented in Figs. 6 and 7.

The GC-MS analysis of the extract revealed several compounds with reported anti-diabetic activity. The major portion of the extract contained mome-inositol. In a study, GC-MS analysis of Bauhinia racemosa leaves revealed the presence of mome-inositol and the extract exhibited In-vitro anti-diabetic activity [23]. Similarly, in another study, GC-MS analysis of a methanolic extract of Decalepis hamiltonii revealed the presence of mome-inositol as a major compound and exhibited strong anti-diabetic activity as revealed by in-silico analysis [24]. The extract also contains 1,3,5-triazine-2,4,6-triamine and D-allose, the compounds with proven anti-diabetic activity [25,26]; octadecanoic acid, a similar compound present in Carica papaya extract which demonstrated to ameliorate obstructive liver dysfunction in diabetic rats [27]; 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, a compound with proved anti-oxidant activity [28]; 3,4-dimethyltetrahydrofuran; and a compound 2,5-bis-aryl-3,4-dimethyltetrahydrofuran present in Myristica fragrans has been reported to exert anti-obesity effect via modulation of AMP-activated protein kinase (AMPK) [29]; neophytadiene, which is reported to have good anti-oxidant activity [30]. All these compounds present in the extract may be responsible for its anti-diabetic, anti-oxidant, and lipid-lowering properties.
Fig. 4. Effect of various treatments on lipid profile of experimental rats

Fig. 5. Effect of various treatments on liver enzymes of experimental rats
CONCLUSION

The GC-MS analysis of hydroalcoholic leaves extract of Cissampelos pareira revealed the presence of some anti-diabetic, anti-oxidant, and anti-obesity compounds such as mome-inositol, 1,3,5-traizine-2,4,6-triamine, D-allose, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one,

Fig. 6. Effect of various treatments on GSH level of experimental rats

Fig. 7. Effect of various treatments on MDA level of experimental rats
2,5-bis-aryl-3,4-dimethyltetrahydrofuran, neophytadiene. The extract with doses 200 and 400 mg/kg acted as a potent anti-diabetic agent against STZ induced diabetes in rats. The effect of extract at dose 400 mg/kg was comparable to standard. Nonetheless, more evidenced-based research is warranted to isolate and characterized these compounds to expand their clinical importance.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

All the animal experiments were in compliance with Animal Ethical Committee, Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA) and were approved by Institutional Animal Ethics Committee (IAEC) of Bilwal Medchem and Research Laboratory Pvt. Ltd., SKS Reengus Industrial Area,Reengus Sikar, (Raj.) (Ethical committee IAEC reg. no. 2005/PO/RcBT/S/18/CPCSEA).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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