INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Assessment of antidiabetic activity of ethanolic extracts of *Gyrocarpus asiaticus* Willd bark against Streptozotocin induced diabetes in rats

Padmaja Yelchuri*, Sudhakar Yajaman

Government Polytechnic for Women, Kadapa,

Andhra Pradesh, India.

ABSTRACT

The principal aim of the investigation was to explore the antidiabetic potential of ethanolic extract of and bark of *Gyrocarpus asiaticus Willd* (Hernandiaceae) (GAE) against streptozotocin induced diabetes in rats. The shade dried leaves were pulverized and extracted by successive extraction in a soxhlator. Phytochemical studies showed that GAE contain significant quantities of alkaloids, flavanoids, saponins, carbohydrates and phytosterols. Results of acute toxicity studies as per OECD guidelines 420 and 425 and subacute toxicity studies as per OECD guidelines 407 suggest that extract can be considered under category 5. In oral glucose tolerance test, GAE at the dose of 400mg/kg, p.o showed significant decrease in elevated blood glucose levels within 30 min. Antidiabetic potential of test extract GAE at three different doses (100, 200, and 400 mg/kg b.w p.o.) was studied on STZ induced diabetic rats for 21 days. Normal control, diabetic control and Standard (Glibenclamide) groups were also conducted in similar fashion. Biochemical parameters such as blood glucose, TG, TC, HDLC, SGOT, SGPT, ALP, glycosylated Hb and weight of animals were noted at 0,7,14 and 21days of anti-diabetic studies in STZ induced rats. Glycogen content in the liver and skeletal muscle was estimated after 21 days of treatment. The test drugs showed progressive ($P<0.001$) reduction in blood glucose, TG, TC, SGOT, SGPT, ALP, glycosylated Hb levels and elevation in HDLC, Liver &muscle glycogen levels and in a dose-related manner during the period of study. The results suggested that the GAE at 400 mg/kg doses (93.6%) after 21 days showed equipotent effect with that of standard drug glibenclamide (97.8 %). The IR, ^{1}H NMR, ^{13}C NMR, and Mass spectral analysis of GAE_1, GAE_2 and GAE_3 were found to be 2(-) Tetrahydro palmatine, Stigmasterol and Kaempferol.

Key words: Antidiabetic, *Gyrocarpus asiaticus*, ethanolic extract, glibenclamide, streptozotocin.

INTRODUCTION

Diabetes mellitus, a chronic endocrine disorder characterized by metabolic derangements of carbohydrate, fat and protein, develops over a period of time into nephropathy, neuropathy, retinopathy, cardiomyopathy etc (Arky, 1982). Though many synthetic hypoglycemic agents were synthesized for maintenance of type 2 diabetes in the recent past, the diabetes and the related complications continued to be major medical problem throughout the world. As the prevalence of diabetes mellitus is estimated to be more than 300 million by 2025 (Ojewoleet al., 2006), the WHO recommended the evaluation of traditional plant treatments for management of diabetes considering their effectiveness, non-toxicity and suitability for oral therapy (Patel & Srinivasan, 1997). It is relevant to India as several recent surveys documented increased ethnic susceptibility to diabetes in Indians (Ramaiya et al., 1991). Indian medicinal plants used in folklore medicine are very effective in the maintenace of diabetes (Nadkarni, 1976; Kirithikar&Basu, 1995).

Gyrocarpus asiaticus Willd is a member of the Hernandiaceae family was described by Nicolaas Joseph von Jacquin in 1763. It is found all around the Pacific region (South America, Papau New Guinea, Australia and more) and Asia. It grows in a well drained peat with some water and lots of sun. It can be reproduced by seeds and probably by cuttings. The flowers are small and whitish to yellowish-green, and male and bisexual flowers grow in the same flower-head. The fruits are olive-shaped, large, tough, woody, dry, and blackish. The seeds are slightly winged. The bark collected in the month of April was used in the present investigation.

MATERIALS AND METHODS

Plant material

The plants were authenticated by Dr. B.Prathibha Devi, Professor & Head, Department of Pharmacy, Osmania University, Hyderabad. A.P. and the specimens have been preserved in our research lab (GPWKDP/2009/YP/01&02).

Extraction

Shade dried crude drug was pulverized, sieved (10/44) and stored in air-tight containers. About 5000 grams of crude drug was extracted using AR grade solvents Petroleum ether (60 – 80°C), Benzene, Chloroform, Acetone, Ethyl acetate and Ethanol (95%) by successive soxhlation method until the phytoconstituents were completely exhausted. The extracts were concentrated by using rota–vacuum evaporator (Buchi type, Mumbai, India) until a semisolid extract is obtained, dried at less than 50°C, comminuted in a ball mill and preserved in air tight containers kept in desiccators prior to its studies and labeled as GAE.

Materials

Streptozotocin was purchased from Sigma-Aldrich, India and the solution was prepared by freshly dissolving in citrate buffer (0.01 M, pH 4.5). Glibenclamide was procured from Cipla Ltd. Diagnostic kits used in this study were procured from Span Diagnostics Ltd., India. All the other chemicals used were of analytical grade.

Phytochemical investigation

Phytochemical tests for the both extracts were carried out (Kokate, 1994) to find out the presence of phytoconstituents viz alkaloids, flavonoids, phytosterols, phenolic compounds, carbohydrates, tannins, triterpenoids etc. and the results were given in Table 1.

PHARMACOLOGICAL STUDIES

Acute toxicity studies: OECD Guidelines No. 420

Female wistar rats (nulliparous and non-pregnant) of 8 to 10 weeks old weighing 200 – 250gms supplied by National Institute of Nutrition, Hyderabad, India, were individually housed in polypropylene cages lined with husk renewed every 24 h in well-ventilated rooms at 22±3°C and RH between 50 to 60, under artificial lighting12:12 h light and dark cycle in hygienic condition for at least five days prior to the study. The rats were fed with standard laboratory pellet diet (Hindustan lever) and water *ad libitum*. The studies were performed according to OECD Guidelines 420 and the protocol was approved by the Institutional Animal Ethics Committee (Reg. No. VNCP/1472/PO/a/CPCSEA).

Sighting study

Animals were fasted over-night prior to dosing and weighed. The test substance was administered to single animals in a sequential manner following the flow charts in Annex 2 of OECD 420. The starting dose for the sighting study was selected from the fixed dose levels of 300 mg/kg (as there is no evidence from *in vivo* and *in vitro* data). The next dose used for this study was 2000 mg/kg. The Test substances were administered in a constant volume of 2 mL/100g body weight in the form of suspension. After the substance has been administered, food was withheld for a further 3-4 h. A period of at least 24 hours was allowed between the dosing of each animal. All animals were observed for at least 14 days.

Main study

A total of five female wistar rats were used for each dose level investigated and the animals were made up of one animal from the sighting study dosed at the selected dose level together with an additional four animals. The time interval between dosing at each level was 3 or 4 days.

Acute toxicity studies: OECD Guidelines No. 425

Animals were divided into two groups of 3 animals each. Group I was treated with vehicle (distilled water) and was kept as a control. Group II was treated with 5000 mg/kg dose according to their body

weight. Blood and tissue were collected on 14th day. Hematological and biochemical parameters were measured in treated group as well as in control group. The organs were quickly blotted and weighed in a digital balance. Gross necropsy of heart, liver and kidney were observed.

Sub acute toxicity studies: OECD Guidelines No. 407

The plant extract at the dose of 250, 500 and 1000 mg/kg body weight were administered orally to 4 groups of six rats respectively to every 24 h for 28 days and control received vehicle at the same volume. The toxic manifestation such as body weight, mortality, and food and water intake was monitored. After 28 days all surviving animals were fasted overnight and anaesthetized with ether. The heparinised blood samples were collected for determining haematological parameters and the serum from non-heparinised blood was carefully collected for determining clinical blood chemistry. Animals were sacrificed after blood collection and the internal organs were removed and weighed to determine the relative organ weights and observed for gross lesions. The internal organs were preserved in 10% buffered formaldehyde solution for histological examination.

Oral Glucose Tolerance Test for GAE

Wistar rats of either sex weighing 200 – 250gms obtained from National Institute of Nutrition, Hyderabad, India, were individually housed in polypropylene cages lined with husk renewed every 24 h in well-ventilated rooms at 22±3°C and RH between 50 to 60, under artificial lighting12:12 h light and dark cycle in hygienic condition for at least five days prior to the study. The rats were fed with standard laboratory pellet diet (Hindustan lever) and water *ad libitum*. The protocol was approved by the Institutional Animal Ethics Committee.

The animals were divided into five groups of six rats in each group.

Group-I: Glucose (2 g/kg p.o. b.w.)

Group-II: Glibenclamide (0.5 mg/kg) and glucose (2 g/kg p.o. b.w.).

Group-III: GAE (100 mg/kg) and glucose (2 g/kg p.o. b.w.)

Group-IV:GAE (200 mg/kg) and glucose (2 g/kg p.o. b.w.).

Group-V: GAE (400 mg/kg) and glucose (2 g/kg p.o. b.w.).

The animals were fasted overnight and treated with above dosage schedule orally. The GAE and glibenclamide were administered half an hour before administration of glucose solution. Blood glucose levels were determined at 0 (before glucose challenge) 30, 90, 150th min after glucose administration. Serum was separated and glucose levels were measured immediately. The results were given in Table 2.

ANTIDIABETIC ACTIVITY ON STREPTOZOTOCIN INDUCED DIABETIC RATS

Wistar rats of either sex weighing 200 – 250gms obtained from National Institute of Nutrition, Hyderabad, India, were individually housed in polypropylene cages lined with husk renewed every 24 h in well-ventilated rooms at 22±3°C and RH between 50 to 60, under artificial lighting12:12 h light and dark cycle in hygienic condition for at least five days prior to the study. The rats were fed with standard laboratory pellet diet (Hindustan lever) and water *ad libitum*. The protocol was approved by the Institutional Animal Ethics Committee.

Experimental Protocol

About 50 animals as described above were selected for the experiment; six animals were kept separately as normal control group (Group I). Remaining 44 animals were made diabetic by a single intraperitoneal injection of Streptozotocin (50 mg/kg of body weight) dissolved in citrate buffer (0.01 M, pH 4.5).The blood glucose level was checked before and 72 h after STZ injection to confirm the development of diabetes. The diabetic animals were stabilized for five days and the next day (day 0) experiment was started. Only those animals which showed blood glucose levels >250 mg/dL were separated and used for the study (Kumar. S. et al.. 2012) The rats were provided with 5% glucose solution bottles in their cages for the next 24 h to prevent hypoglycaemia (Stanley et al., 1998).

The rats were divided into six groups each consisting of six rats.

Group-1: Normal control - Administered 1%w/v sodium CMC p.o.

Group-2: Diabetic control - Administered STZ (50 mg/kg, i.p.).

Group-3: STZ (50 mg/kg, i.p.) induced diabetic animals administered glibenclamide 0.5 mg/kg p.o. once daily for 21 days

Group-4: STZ (50 mg/kg, i.p.) induced diabetic animals administered GAE 100 mg/kg in 1%w/v sodium CMC p.o. once daily for 21 days.

Group-5: STZ (50 mg/kg, i.p.) induced diabetic animals administered GAE 200 mg/kg in 1%w/v sodium CMC p.o. once daily for 21 days.

Group-6: STZ (50 mg/kg, i.p.) induced diabetic animals administered GAE 400 mg/kg in 1%w/v sodium CMC p.o. once daily for 21 days.

Biochemical estimations

Blood samples were collected from the animals prior to the treatment with above schedule and after 30 min of Glibenclamide/test drug administration on 7th, 14th and 21st day. Blood obtained from the retro orbital venous plexus of rats under ether anesthesia using a glass capillary tube, centrifuged (2,500 rpm/10 min) to separate serum. The serum was used for biochemical estimation of blood glucose, triglycerides, Total cholesterol, HDL-cholesterol, Glycosylated haemoglobin, SGOT, SGPT and ALP.

Collection of organs

After 21 days of daily feeding of the test and the standard drugs orally, the animals were euthanized by overdose of ether anaesthesia. Liver and skeletal muscle tissue samples were collected for the assessment of glycogen content.

Analysis

Plasma glucose

Glucose levels were estimated by commercially available glucose kits (Span Diagnostics Ltd, Surat, India) based on glucose oxidase method. The results were given in Table 5.

Estimation of serum total cholesterol (TC)

The total cholesterol was estimated by the one step method (Wybenga et al., 1970). The results were given in Table 3.

Estimation of HDL-cholesterol

HDL-cholesterol level was determined by the commercially available reagent kit (Erba Mannheim, Transansia biomed &Daman, India) based on phosphotungustate method (Herbert, 1984). The results were given in Table 3.

Estimation of serum triglycerides (TG)

The serum triglyceride level is estimated by the method Enzymatic GPO (Werner et al., 1981). The results were given in Table 3a.

Estimation of SGOT and SGPT

Serum transaminase activity was measured according to the method of Reitman and Frankel(1957). The results were given in Table 4.

Estimation of ALP

Serum alkaline phosphatase was estimated by following the method of Kind and King`s (1976). The results were given in Table 4a.

Estimation of Glycosylated haemoglobin

Blood glycosylated hemoglobin was measured using spectrophotometric method. (Tinder P. 1969). The results were given in Table 5.

Glycogen content

Glycogen content in the liver/skeletal muscle tissue was estimated by the method of Plummer et al., 1978 and the glycogen content was expressed as g/g of tissue. The results were given in Table 6.

Weight of animals

The results were given in Table 6.

Statistical analysis

Data were expressed as mean ± SEM, (n=6). Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. Values were considered statistically significant when at $p<0.05$.

RESULTS AND DISCUSSION

Phytochemical studies

Table 1 represents the results of phytochemical analysis of the ethanolic extract of *Gyrocarpus asiaticus* Linn (Hernandiaceae) (GAE). The plant showed the presence of alkaloids, flavonoids, glycosides, phenolic compounds, proteins, saponins, and phytosterols in varied quantities in appreciable amounts. As many as 150 plants having flavanoids, steroids and tannins as active principles were found to possess antidiabetic potential (Pusparaj et al., 2000; Meiselman et al., 1976; Choi et al., 1991; Ernmenisogiu et al., 1995). Hence the extract was selected for antidiabetic studies.

Acute oral toxicity studies

Results of Acute toxicity studies of GAE were conducted as per OECD guidelines 425. The GAE did not show any sign and symptoms of toxicity or mortality up to 5000 mg /kg body weight on oral administration, thus these extracts could be considered as category 5. Body weight before and after administration were noted and any changes in skin, fur, eyes, mucous membranes, respiratory, circulatory, autonomic, central nervous system, somatomotor activity, behavioral pattern, sign of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma were observed. The onset of toxicity and signs of toxicity were not seen in the rats upto 72

hr of observation period. This indicates the safety of extract. Hence, the 100, 200 and 400 mg/kg dose were selected for the further study.

Effect on oral glucose tolerance

Table 2 represents the results of oral glucose tolerance test. The blood glucose levels in the control group (G-I) increased to peak level at 30 min after glucose load and decreased to near normal at 150 min. Group-II (glibenclamide 0.5 mg/kg, p.o) and Group V (GAE 400mg/kg, p.o) showed significant decrease in elevated blood glucose levels at 30 min. Group III and IV showed significant decrease in blood glucose levels at 90 min. Results also suggest that the GAE have not decreased the blood glucose levels below normal levels.

Antidiabetic Activity of GAE

Effect on serum glucose levels

Table 5 represent the results of the study of antidiabetic effect of GAE on STZ induced diabetic rats. The diabetic control group (G-II) showed a significant increase in the serum glucose levels on the 7^{th} , 14^{th} and 21^{st} day in comparison to the normal control (G-I) indicating STZ induced persistent diabetes mellitus. Significant decrease in blood glucose levels on the 7^{th}day in Standard group (G-III) administered glibenclamide, 0.5 mg/kg, p.o, once daily. The serum glucose levels reached to near normal on day 14 and 21.

Similarly in groups G-IV to G-VI (GAE 100, 200 and 400 mg/kg, p.o, once daily), a significant and dose dependent decrease in the serum glucose levels on 7th, 14th and 21^{st}day was observed. G-VI showed equipotent activity with G-III. The plausible mechanism behind the antidiabetic potential of GAE is due to the presence of flavanoids, steroids and tannins which may have increased the activity of enzymes responsible for utilization of glucose by insulin-dependent pathway or regenerate β -cells in pancreatic islets. (Lenzen and panten, 1988; Ahmed et al., 1991; Jorns et al., 1997; Sakurai et al., 2001).

Effect on biochemical parameters

Serum levels of TC, TG, SGOT, SGPT, ALP, Glycosylated haemoglobin were decreased progressively after 21 days of treatment in a dose-related manner. It is also observed that HDL-Cholesterol levels were increased progressively during the period of study.

In this study, the GAE at 400 mg/kg p.o significantly decreased TC (82.85%), TG (84.03%), SGOT (85.2%), SGPT (93.2%), ALP (95.6%), Glycosylated haemoglobin (82.9%) and significantly increased HDL- cholesterol level (35.63%).

Liver & muscle glycogen contents were increased significantly after 21 days of treatment of test extracts in a dose-related manner. Similarly, the weight of the animals also gained to normal in the treated animals.

The increase in HDL-cholesterol levels is very beneficial because of its protective effects in prevention of cardiovascular diseases. Since STZ permanently destroys the pancreatic β cells, lowering of blood glucose level in Streptozotocinised rats after administration of the extracts indicates that the extract possesses extra pancreatic effects (Bhupesh et al., 2008). On the basis of the above evidences, the antidiabetic activity of GAE may be due to presence of flavanoids, steroids and tannins (Iwu, 1983; Iwu, 1980).

ISOLATION OF PHYTOCHEMICAL CONSTITUENTS

Preparation of sample:

Sample preparation is the most important step in the development of analytical methods for the analysis of botanicals and herbal preparations. The ethanolic extract of the test extract (GAE) were fractioned by extraction with petroleum ether (40°- 60°C) (fraction-I), ethyl ether (fraction-II) and ethyl acetate (fraction-III) in succession. Each of the steps was repeated thrice to ensure complete extraction. Fraction III of each of the test samples was hydrolysed by refluxing with 7% H_2SO_4 (10 ml/gm residue) for 5 hours. The mixture was filtered and the filtrate was extracted thrice with ethyl acetate in a separating funnel. All the ethyl acetate layers were mixed, washed with distilled water several times until neutrality is attained, and then concentrated *in vacuon* and then subjected to chromatographic examination.

Preparation of column:

A glass column (60cmX6cm), stationary phase (Silica gel G (0.2-0.3 mm thick) and mobile phase (Ethanol: Ammonium hydroxide, 200 : 1) was used for the isolation procedures. The glass column was cleaned thoroughly in water and then rinsed with acetone and fixed vertically in a stand. A wad of glass wool is placed at the bottom of the column to prevent the passage of solid support. Slurry of silica gel G was prepared with the mobile phase in a beaker and poured into the glass column slowly to ensure even packing. 2 -3 cm of solvent system is always maintained at the top of the column to avoid the drying. Fresh solvent system Ethanol: ammonium hydroxide, 200:1 is poured on the top of the column and eluted once; 2 cm of solvent system is maintained at the top of the column.

Sample loading:
20 gms of the sample was weighed and mixed with silica gel G and kept air dried, the dried material was loaded on the top of the column and a wad of glass wool was placed above it to prevent the disturbance of solvent addition. Now the mobile phase was poured slowly at the top of the column and observed for the separation. The flow rate was adjusted to 30 drops per minute. 50 ml each of the eluents were collected in small beakers. 121 such fractions were collected and the volumes of the eluents were reduced by evaporation.

TLC Profiles of the eluents
Thin layer chromatographic characterization has been performed for all the fractions individually. Sample from each fraction was separately applied 1 cm above the edge of the TLC plates (Merck) along with standard reference samples. These plates were developed in an airtight chromatography chamber containing about 200 ml of solvent mixture of benzene, acetic acid and water (125:72:3). The developed plates were air dried and visualized under UV light after exposure to ammonia fumes by placing at the mouth of bottle containing concentrated ammonium hydroxide held in contact with each spot for about 5-10 seconds. Fluorescent spots corresponding to that of standard markers were marked. The developed plates were sprayed with 5% ferric chloride solution, 0.1% alcoholic $AlCl_3$ and kept in I_2 chamber to observe the colour of the spots. Rf values were calculated for isolated samples and compared with coinciding standard. The fractions showed similar hRf values were mixed together. The combined fractions having similar hRf value were subjected to the separation process.

Preparative thin layer chromatography (PTLC)
Glass plates (20 x 20 cm) thickly coated (0.4-0.5 nm) with silica gel 'G' (BDH, 500 µ thick) and activated at 100°C for 30 minutes and cooled at room temperature were used for preparative thin layer chromatography (PTLC). The combined fractions having hRf values were applied on separate plates and the plates were developed in an airtight chromatography chamber containing about 200 ml of solvent mixture of benzene, acetic acid and water (125: 72:3), air dried and visualized under UV light. Each of the spots coinciding with those of standard reference compounds was marked. The marked spots were scrapped and collected separately along with the silica gel 'G' and eluted with ethanol.

Purification of the eluent
Eluted fractions were filtered, dried and again cochromatographed with standard markers. The marked spots were scrapped and collected separately along with the silica gel 'G' and eluted with ethanol. The eluent was subjected to repeated filtration through whatman filter paper saturated with ethanol to ensure complete separation from silica gel G. The fraction was left air dried, the residue was collected and subjected to single crystal growth.

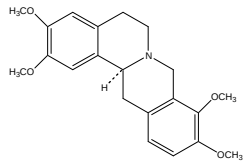
Procedure for obtaining single crystal:
The residue obtained was completely dissolved in HPLC grade Ethanol, and single crystal development was assisted by slow diffusion of benzene to this solution, stoppered tightly and kept undisturbed. After 7 days, the crystals observed were gently removed from the mother liquor and washed with benzene, weighed and packed individually in separate vials.

Analysis of isolated compounds
Physico chemical properties of the isolated material:
Physical properties such as Colour, odour, melting point, solubility in different solvents were recorded. Chemical nature of the compounds was also recorded by subjecting to different qualitative chemical tests. The IR spectra of the isolated compounds were recorded in JASCO – FTIR Spectrophotometer using potassium bromide disc in the region 2000cm^{-1} to 200cm^{-1}. The ^{1}H NMR, ^{13C} NMR spectrums of the isolated compounds was recorded in Bruker DPX-300 NMT Spectrometer using $CDCl_3$ as solvent and respective internal standards. Mass spectrums of isolated compounds were recorded in JEOL JMS 600 in FAB mode.

Structural elucidation of isolated compounds
GAE_1 was isolated as single colourless crystal. The IR spectra in KBr revealed the presence of C=C : 1610.27, 1513.0 cm^{-1}, C-N : 3610.0 cm^{-1}, C-O : 1280.0, 1257.0 cm^{-1} . The data of ^{1}HNMR (DMSO-d_6) revealed the Presence of 25 protons. Four different type of Protons were observed. Aromatic CH 04 04 δ 6.77, δ6.73, δ 6.86, δ6.89, Methylene CH2 04 08 δ 2.60, δ 3.16, δ2.69, δ3.28 δ3.24, δ2.87 , δ2.71, δ2.64 Methine H 01 01 δ4.267 Free Methyl CH3 04 12 δ 3.53, δ3.56, δ3.51, δ δ3.77, δ 3.79 δ3.81, δ3.85, δ 3.86, δ3.89, δ3.92, δ3.93, δ3.95, δ 3.29. From Mass spectral data the molecular weight of compound was observed to be 355. The Molecular ion peak was observed at m/z 355, The Base peak was observed at m/z 354, The Isotopic peak were

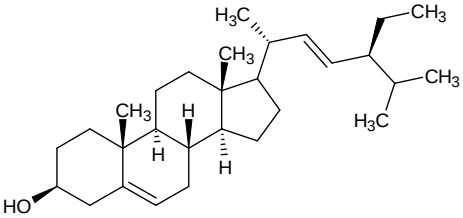
observed at m/z 356, m/z 357. All the recorded data such as IR, 1HNMR and mass spectrum conclusively prove that the isolated compound may be "2. (-)-tetrahydro palmatine"

2. (-)- tetrahydro palmatine



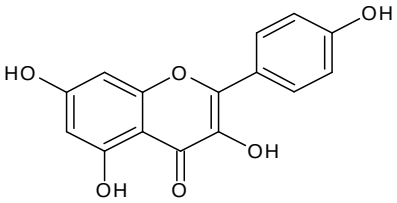
2,3,9,10-Tetramethoxy-5,8,13,13a-tetrahydro-6*H*-isoquino[3,2-*a*]isoquinoline

GAE_2 was isolated as white fine particles. The mass spectral data of the compound provided a molecular formula C_{29} H_{48} O, which was supported by the ^{13}C NMR spectral data. ^{1}H NMR spectra of GAE_1 revealed the presence of two methyl sinlgets at δ 0.71, and 1.03; three methyl doublets that appeared at δ 0.80, 0.82, and 0.91; and a methyl triplet at δ 0.83. GAE_2 also showed protons at δ 4.98, 5.14, and 5.31 suggesting the presence of three protons corresponding to that of a trisubstituted and a disubstituted olefinic bond. Liebermann-Burchard reaction indicated GAE_2 is having a sterol skeleton**.** The proton corresponding to the H-3 of a sterol moiety was appeared as a triplet of doublet of doublets at δ 3.51. The above spectral data reinforced the presence of sterol skeleton having a hydroxyl group at C-3 position with two double bonds at C-5/C-6 and C-20/C-21 with six methyl groups which was supported by the key COSY and HMBC correlations. Thus, the structure of GAE_1 was assigned as the known compound Stigmasterol.



GAE_2 - Stigmasterol

The IR spectrum showed characteristic peak positions of active ingredients- GAE_3 3411 cm^{-1} : O-H stretching vibration of phenols, 1663.8 cm^{-1} : C=O Aryl Ketonic stretch, 1608.8cm^{-1}, 1523.5cm^{-1} , 1496cm^{-1} : -C-C-Aromatic ring stretch, 1383.1cm^{-1} : Inplane bending of C-H bond in Aromatic Hydrocarbon , 1265cm^{-1} : C-O stretch of Aryl ether ,1203cm^{-1}: C-O stretch of Phenol, 1167: C-CO-C stretch and bending in ketone, 940.6, 821.4, 677, 602.3cm^{-1}: out of plane C-H bending of aromatic hydrocarbon group of alcohol respectively, present in the molecular structure of GAE_3. From the mass spectral data of the GAE_3, the molecular formula was found to be C_{15} H_{10} O_7 which was supported by the ^{13}C NMR spectral data.



GAE_3 – Kaempferol

The IR, ^{1}H NMR, ^{13}C-NMR and MS spectral data suggests that GAE_1 may be "2. (-)- tetrahydro palmatine", GAE_2 may be "Stigmasterol", GAE_3 it may be "Kaempferol".

CONCLUSION

The ethanolic extract of *Gyrocarpus asiaticus* at the dose of 400 mg/kg body weight showed a significant antidiabetic activity and its efficacy is on par with the standard drug, Glibenclamide 0.5 mg/kg, p.o. This may be due to the presence appreciable quantities of phytoconstituents such as flavanoids, glycosides, phenolic compounds, proteins, saponins, Proteins, Triterpenoids and steroids. One possible mechanism of action postulated is stimulation of insulin secretion there by improvement of glycogenesis process. Further studies are in progress to isolate the active principle(s) in the extracts as well as to elucidate their exact mechanism(s) of action. As the extracts are found to be considered as potentially safe i.e. category 5 as per the OECD guidelines 425, and showed equipotent antidiabetic efficacy, these extracts may be used as alternative for patients in the management of type 2 diabetes mellitus. The IR, ^{1}H NMR, ^{13}C NMR, and Mass spectral analysis of GAE_1, GAE_2 and GAE_3 confirmed the compounds as 2. (-)- tetrahydro palmatine, Stigmasterol, Kaempferol.

Table 1: Phytochemical analysis of extracts of *Gyrocarpus asiaticus* Willd

Constituent	GAP	GAB	GAC	GAA	GAEA	GAE
Alkaloids						
Mayer`s test	-	+	++	++	++	+++
Dragendorff`s test	-	+	+	+	+	+++
Wagner`s test	-	+	+	+	+	+++
Hager`s test	-	+	+	+	+	+++
Carbohydrates						
Molish`s test	+	+	+	+	+	+++
Fehling`s test	+	+	+	+	+	++
Benedict`stest	+	+	+	+	+	++
Glycosides						
Borntrager`s test	-	-	-	-	-	-
Saponins						
Foam test	+	+	+	+	+	+++
Flavonoids						
Ferric chloride test	+	+	+	+	++	++
Shinoda test	+	+	+	+	+	+++
Lead acetate test	+	+	+	+	+	+
Alkaline reagent test	+	+	+	+	++	++
Steroids						
Libermann-Burchard test	+	+	+	+	+	+
Salkowski test	+	+	+	+	+	+
Proteins						
Millon`s test	++	++	++	++	++	+++
Biuret test	+	+	+	+	+	++
Ninhydrin test	+	+	+	+	+	+++

+ Positive, - Negative

Table 2: Effect of GAE on Oral glucose tolerance test

Group	Blood glucose levels (mg/dL)			
	Initial	**30 min**	**90 min**	**150 min**
I	83.67 ±0.33	139.2 ± 1.10	127.9 ±1.66	89.50 ± 1.05
II	79.83 ± 0.60	119.5 ± 0.76	86.0 ±1.50***	75.33 ± 1.3***
III	81.52 ± 1.178	132.13 ± 2.39	124.28 ± 2.89**	78.65 ± 2.8*
IV	79.54 ± 1.27	129.11±1.25	106.69 ± 0.739**	81.31 ± 2.697*
V	78.54 ± 1.407	125.11 ± 1.19	95.5 ± 0.397***	77.9± 0.622**

Each value represents the mean ± SEM. n = 6. Values**P<0.001, *P<0.01, #P<0.05.compared to positive control

Table 3: Effect of GAE on serum levels of Total Cholesterol and HDL-Cholesterol.

	TC				HDL-Cholesterol			
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21
Group 1	103.3±4.5	102.1±5.3	103.1±5.6	101.2±5.6	56.2±1.2	56.6±1.5	55.6±1.2	55.9±2.1
Group 2	206.6±5.6	204.5±6.5	205.3±6.4	205.6±5.4	46.6±2.6	48.9±1.6	46.6±1.3	47.2±1.3
Group 3	204.6±6.1	171.4±4.5	139.7±5.9	109.5±3.8	45.4±1.6	48.5±1.6	51.3±1.4	53.9±2.5
Group 4	205.3±7.5	195.7±5.9	177.5±6.4	149.8±6.4	45.1±2.5	45.9±1.2	46.9±1.8	47.5±1.9
Group 5	206.5±6.4	185.6±4.9	159.3±6.7	139.9±6.3	44.3±2.4	46.3±1.4	47.1±0.9	49.7±2.5
Group 6	204.2±8.4	177.2±8.9	149.1±5.7	119.1±3.7	44.8±1.9	45.7±1.7	48.2±1.6	50.3±2.7

Each value represents the mean ± SEM. n = 6. Values**P<0.001, *P<0.01, #P<0.05.compared to positive control

Table 3a: Effect of GAE on serum levels of Triglycerides

	Triglyceride			
	Day 0	Day 7	Day 14	Day 21
Group 1	88.2±5.3	89.1±5.6	90.3±4.6	89.3±4.6
Group 2	136.3±6.5	139.6±6.4	137.6±5.4	136.9±5.5
Group 3	134.6±6.4	121.7±6.8	104.2±5.9	92.4±5.9
Group 4	134.2±6.9	129.9±5.9	119.7±5.8	106.9±5.4
Group 5	135.6±5.6	128.7±5.7	114.7±5.6	96.8±5.6
Group 6	134.8±5.7	123.9±5.1	111.3±5.4	96.9±5.3

Each value represents the mean ± SEM. n = 6. Values**P<0.001, *P<0.01, #P<0.05.compared to positive control

Table 4: Effect of GAE on SGOT and SGPT

	SGOT				SGPT			
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 0**	**Day 7**	**Day 14**	**Day 21**
Group 1	33.3±2.4	34.22±2.5	35.5±2.4	34.5±1.8	35.5±2.5	36.75±3.5	37.5±2.4	34.6±0.1
Group 2	73.5±2.2	74.2±1.6	74.6±2.6	73.8±1.5	134.9±4.6	135.3±4.2	133.6±3.5	134.6±3.5
Group 3	73.9±2.3	61.5±2.4	43.8±2.3	36.1±1.6	136.2±3.5	96.5±3.5	65.5±2.6	37.5±2.4
Group 4	74.4±2.3	69.5±2.4	53.9±0.8	46.9±2.1	133.6±4.3	112.9±4.6	80.1±3.8	46.6±2.9
Group 5	73.9±1.8	67.1±1.8	51.8±2.4	42.6±2.3	134.1±3.5	105.9±4.6	76.9±2.9	43.9±3.1
Group 6	71.5±1.7	64.2±1.8	48.9±2.6	40.3±2.1	135.9±3.8	99.4±3.8	72.9±2.8	41.4±3.5

Each value represents the mean ± SEM. n = 6. Values**P<0.001, *P<0.01, #P<0.05.compared to positive control

Table 4a: Effect of GAE ALP

	ALP			
	Day 0	**Day 7**	**Day 14**	**Day 21**
Group 1	289.5±11.2	292.6±10.2	292.6±10.6	291.3±10.5
Group 2	841.6±15.6	839.6±9.5	842.2±9.5	836.1±9.6
Group 3	845.9±12.5	520.6±9.6	356.5±9.6	298.9±9.4
Group 4	845.6±10.9	551.9±11.9	391.6±9.4	327.8±12.5
Group 5	844.5±16.5	542.1±12.3	379.3±11.3	321.6±9.4
Group 6	844.1±12.1	534.6±10.2	367.1±12.5	315.1±10.2

Each value represents the mean ± SEM. n = 6. Values**P<0.001, *P<0.01, #P<0.05.compared to positive control

Table 5: Effect of GAE on serum levels of Glycosylated haemoglobin and Glucose

	Glycosylated Hb (%)				Glucose (mg/dL)			
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 0**	**Day 7**	**Day 14**	**Day 21**
Group 1	8.1±0.9	9.1±0.9	8.5±1.5	8.7±1.1	115.27±3.5	113.4±2.5	112.4±2.5	114.23±1.9
Group 2	13.5±1.2	12.7±1.5	13.9±1.2	13.4±1.2	272.69±4.5	295.6±3.5	324.1±2.6	351.5±1.8
Group 3	13.9±1.5	11.5±1.3	10.1±1.6	9.1±0.8	281.45±5.6	209.5±3.4	135.2±3.5	119.5±1.7
Group 4	13.5±1.2	13.2±1.2	11.7±1.8	10.2±1.2	281.69±5.4	255.4±3.8	171.2±2.6	151.6±2.7
Group 5	13.7±1.5	12.5±1.6	11.2±1.2	9.9±1.5	279.56±4.8	241.7±3.9	164.5±2.5	137.7±2.6
Group 6	13.6±0.8	12.1±.08	10.9±0.8	9.5±0.9	274.58±4.7	226.7±3.2	157.4±2.7	129.3±2.4

Each value represents the mean ± SEM. n = 6. Values**P<0.001, *P<0.01, #P<0.05.compared to positive control

Table 6: Effect of GAE on Liver glycogen, Muscle glycogen and Weight of the animals

	Glycosylated Hb				Glycogen (mg/g)	
	Day 0	Day 7	Day 14	Day 21	Liver	Muscle
Group 1	8.1±0.9	9.1±0.9	8.5±1.5	8.7±1.1	54.2±1.2	8.9±1.7
Group 2	13.5±1.2	12.7±1.5	13.9±1.2	13.4±1.2	17.6±2.1	3.4±1.3
Group 3	13.9±1.5	11.5±1.3	10.1±1.6	9.1±0.8	51.6±1.9	7.8±2.4
Group 4	13.5±1.2	13.2±1.2	11.7±1.8	10.2±1.2	31.6±2.3	3.9±1.6
Group 5	13.7±1.5	12.5±1.6	11.2±1.2	9.9±1.5	39.5±3.1	5.2±1.8
Group 6	13.6±0.8	12.1±.08	10.9±0.8	9.5±0.9	46.2±2.8	6.9±2.4

Each value represents the mean ± SEM. n = 6. Values**P<0.001, *P<0.01, #P<0.05.compared to positive control

Table No: 6. Isolation of Chemical constituents from GAE

Fraction no.	Weight of Residue (g)	Compound Isolated
1-10	2.230	waxy residue
11-13	2.235	waxy residue
14-20	0..260	greenish coloring matter
21-26	0.114	Colourless powder GAE-1 (passed the test for alkalloids)
27-30	0.254	White powder GAE-2 (passed the test for steroids)
31-39	0.138	waxy residue
40-45	0.526	GAE-3 (passed the test for steroids)
46-55	0.315	greenish coloring matter
56-62	0.470	greenish coloring matter
63-66	0.202	greenish coloring matter
67-71	0.170	Intangible mass
72-78	0.320	Intangible mass
79-84	0.100	Intangible mass
85-89	0.050	Intangible mass
90-98	0.024	greenish coloring matter
99-108	0.236	greenish coloring matter
109-116	0.281	greenish coloring matter
117-121	0.236	greenish coloring matter

REFERENCES

1. Ahmed T, Adeghate E, Sharma AK, Pallot DJ, Singh J. Effect of *Momordicacharantia* fruit juice on islet of morphology in the pancreas of streptozotocin diabetic rat.Diab Res Clinpract1991; 40: 145-151.
2. Banik.G, Bawari.M, DuttaChoudhury.S, Choudhury.S, Sharma.GD. Someantidiabetic plants of Southern Assam. *Assam UnivJourn of Sci and Tech. Biol and EnvSci* 2010; 5(1): 114-119.
3. Bhupesh CS, Kamal S. Antidiabetic activity of steam bark of Berberiesaristata D.C. in alloxan induced diabetic rats. The internet journal of pharmacology,2008; 6:1.
4. Choi JS, Yokozawa T, Oura H. Improvement of hyperglycemia and hyperlipidmia in streptozocin – diabetic rats by methanolic extract of Prunusdavidiane stems and its main component, pruning.Planta Med.1991; 57: 208.
5. Christophe W. (2006).Ethano pharmacology of medicinal plants.Asia and pacific. New Jersey: Humana press.
6. Colca JR. Insulin sensitizers may prevent metabolic inflammation. BiochemPharmacol, 2006;72: 125-131.
7. De Sousa E, Zanatta L, Seifriz I. Hypoglycemic effect and antioxidant potential of kaempferol-3, 7-O-dirhamnoside from *Bauhinia forficate* leaves. J. Nat. Prod, 2004;67: 829-832.
8. Ernmenisogiu A, Kelestimur F, Koker AH. Hypoglycemic effect of *Ziziphus Jujube* leaves. J Pharm pharmacology.1995;47:72.
9. Herbert K.(1984). Lipids, In Clinical Chemistry: Theory, Analysis and co-relation: Mosby, Toronto, 1182-1230.
10. Iwu MM. Antidiabetic properties of *Beridelia furruginear* leaves. Plant Med. 1980;39: 24.
11. Iwu MM. Hypoglycemic property of *Beridelia furruginear* leaves. Fitoterapia.1983;54:243.
12. Jorns A, Munday R, TiedgeM,Lenzen S. Comparative toxicity of alloxan, N-alkyl-alloxans and ninhydrin to isolated pancreatic islets *in- vitro*. J Endocrinol, 1997;155: 283-293.
13. Jose JK, Kuttan G, Kuttan R. Antitumour activity of *Emblicaofficinalis*. J.Ethnopharmacol. 2001;75: 65-69.
14. Kaleem M, Asif M, Ahmed QU,Bano B. Antidiabetic and antioxidant activity of *Annona squamosa* extract in streptozotocin-induced diabetic rats. Singapore Med. J, 2006;47: 670.
15. Kaplan A, Lavernel LS. Disorder of Carbohydrate, In-Clinical Chemistry, Interpretation and Techniques: Lea and Febiger. 2nd edtn, Philadelphia,1983: 301-320.
16. Kaplan LA, Pesce AJ, C.V. Mosby, Toronto. Carbohydrates and Metabolite, In clinical chemistry, 1984:1032-1040.
17. Kirithikar KR, Basu B. Indian Medicinal Plants. International book distributors, Dehradun, India:1995; 1: 371 – 372.
18. Kokate CK **(**1994), Practical Pharmacognosy, 4th Ed, 107-112.
19. LenzenS,Panten U. History and mechanism of action.Diabetologia, 1988;31:337-342.
20. McGowan, M.W., Artiss, JD Strandbergh DR, Zak B. A peroxidase-coupled method for the colorimetric determination of serum triglycerides, Clin Chem.1983;29:538-542.
21. Meiselman HL, Halperrn BP, Dateo GP. Reduction of sweetness judgement by extracts from the leaves of *Ziziphus jujube* Physiology and Behavior.1976;17:313.
22. Melendez PA, Capriles VA. Antibacterial properties of tropical plants from Puerto Rico.Phytomedicine, 2006:13 (4), 272 – 276.
23. Nadkarni KM, Nadkarni AK. (1976). Indian MateriaMedica, Vol.1, Popular Prakashan, Bombay, India: 615-616.
24. Nicholas V, Carroll, Robert W, Longley, Joseph H. (1955). The determination of glycogen in liver, and muscle bye use of anthrone reagent.The Journal of Biological Chemistry.
25. Nishikant AR, Naresh JG. Antidiabetic activity of hydroethanol extract of *Cyperus rotundus* in alloxan induced diabetes in rats. Fitoterapia,2006; 77: 588.
26. Ojewole JAO, Adewole SO, Olayiwola G. Hypoglycemic and hypertensive effects of *Momordica charantia* Linn.(Cucurbitaceae) 1036 Afr. J. Pharm. Pharmacol.Whole-plant aqueous extract in rats.Cardiovasc. J. S. Afr, 2006;17(5): 227-232.
27. Pathak RM, Ansari S, Mahmood A. Changes in chemical composition of intestinal brush border membrane in alloxan induced chronic diabetes. Indian J Exp. Biol, 1981;19: 503-505.
28. Plummer DT (1978).Isolation and assay of glycogen from the liver and skeletal muscles of rats.In an Introduction to practical biochemistry, New York MCG Graw – Hill, 182-184.
29. Pusparaj P, Tan CH, Tan BHK. Effects of *Averrhoabi limbi* leaf extract on blood glucose and lipids in streptozotocin diabetic rats. J Ethnopharmacology.2000;72:69.
30. Rajalingam R, Srinivasan N, Govindarajulu P. Effect of alloxan induced diabetes on lipid profiles in renal cortex and medulla of mature albino rats. Indian J Exp. Bial, 1993;31: 577-579.

31. Sakurai K, Katoh M, Someno K, Fujimoto Y. Apoptosis and mitochondrial damage in INS-1 cells treated with alloxan. Biological and pharmaceutical Bulletin, 2001;24: 876-882.
32. Seilfter S, Dayton S, Novic B, Muntwyler E. Arch. Biochem1950; 25: 191.
33. Stanley Mainzen Prince, P., Menon, V.P., Pari, L. Hypoglycaemic activity of *Syzigium cuminii* seeds: effect on lipid peroxidation in alloxan diabetic rats. Journal of Ethnopharmacology, 1998;61: 1 -7.
34. Verspohl EJ. Recommended testing in diabetes research. PlantaMedica, 2002;68: 581-590.
35. Wybenga, D. R. Pileggi, V. J. Dirstine, P. H., Giorgio J D. Direct Manual Determination of Serum Total Cholesterol with a Single Stable Reagent, ClinChem, 1970;16: 980-984.
36. Zanatta L, De Sousa E, Cazarolli LH. Effect of crude extract and fractions from *Vitexmegapotamica* leaves on hyperglycemia in alloxan diabetic rats. J. Ethnopharmacol, 2007;109:151-155.
37. Zimmermann M. Ethical guidelines for investigation of experimental pain in conscious animals.*Pain*, 1983;16: 109-10.