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Antioxidant and Antidiabetic Potentials of *Calotropis gigantea* in RIN-5F Pancreatic Cell Lines

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ABSTRACT

Medicinal plants are potentially important source for the development of novel chemotherapeutic agents. *Calotropis gigantean (C.gigantea)* is a common wasteland weed and known for various medicinal properties. The present study investigates the analysis of major bioactive constituents of medicinally important plant *C.gigantea* in different extract of leaves, flowers and stems. The various organic solvents used for the extraction, subjected to qualitative phytochemical screening for the presence of bioactive ingredients. The analysis shows the presence of many biologically active molecules such as carbohydrates, proteins, amino acid, saponin, tannin, flavanoids, alkaloids, glycosides and polyphenol. The quantitative analysis of total flavanoids content (TPC), tannin content (TTC), and poly phenol content (TPC) were carried out. Ethanolic extract of flower showed highest quantity of identified bioactive compounds and their characteristics of the bioactive constituents were identified by means of gas chromatography coupled with mass spectrometry (GC-MS). The GC-MS result confirms the presence of thirty six compounds in ethanolic flower extract of *C.gigantea.* Further the *in vitro* antioxidant properties of the *C.gigantea* flowers were evaluated by various antioxidant assays, including 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Thiobarbituric acid (TBA) method, Superoxide Anion Radical scavenging assay, Metal chelating activity and Phospho molybdenum assay, assay of CAT, SOD, GPx, GSH. The results showed the presence of potential antioxidants. The antidiabetic effect of ethanolic extract from *C.gigantea* flowers on RIN-5F pancreatic cell line and normal cell lines was evaluated by MTT assay. From the performed assay, the flowers of *C.gigantea* shows greater activity on RIN-5F cell line and little activity on normal cell line. The findings of the present study suggested that the flower extract from *C.gigantea* could be a potential natural source of antioxidants and could have greater importance as therapeutic agent in preventing or slowing diabetic activity that mean extract of *C.gigantea* flowers can be used as antidiabetic agents.

1.0 INTRODUCTION

Diabetes mellitus is a chronic condition characterized by major dearrangements in glucose metabolism and abnormalities in fat and protein metabolism. Plant-based medicinal products have been known to man since ancient times, (1). Plants have been the primary source of drugs and many of the currently available drugs have been directly or indirectly derived from plants. For example, the popular hypoglycemic drug glucophage (metformin) is derived from Galega officinalis (2). About 800 plant species have been reported to possess antidiabetic properties. A wide array of plant derived principles belonging to compounds mainly alkaloids, glycosides, galactomannan gum, polysaccharides, hypoglycans, peptidoglycans, guanidine, steroids, glycopeptides, and terpenoids have demonstrated bioactivity against hyperglycemia. Alkaloids, tannins, saponins, anthraquinones, coumarins, steroid, terpenoid, flavonoids, phlobatannin and cardic glycoside distribution in different medicinal plants (3).

Calotropis is a genus of flowering plants in the dogbane family, Apocynaceae. They are commonly known as milkweeds because of the latex they produce. *Calotropis* species are considered common weeds in some parts of the world. The flowers are fragrant and are often used in making floral tassels in some mainland Southeast Asian cultures. Fibers of these plants are called madar or mader. The plant is known as aak in , and was used in cases of cutaneous diseases, intestinal worms, cough, ascites, asthma, bronchitis, dyspepsia, paralysis, swellings, intermittent fevers, anorexia, inflammations and tumors. In large doses, Arka is known to act as a purgative and an emetic (4).

Calotropis gigantea (C.gigantea) belongs to the family Asclepiadaceae and is a well known Indian medicinal plant locally known as “Akado”. Medicinally important parts of this plant are flower, terminal leaf pairs, root with root bark and latex. *C.gigantea* (Crown flower) is a xerophytic, erect shrub, growing widely throughout the tropical and subtropical regions of Asia and Africa. This plant is popularly known because it produces large quantity of latex (5). Fractionation of the latex into its rubber and rubber-free fractions affords better insight into its potentials and limitations. A large quantity of latex can be easily collected from its green parts. The abundance of latex (containing alkaloids) in the green parts of the plant reinforces the idea that it produced and accumulated latex as a defense strategy against organisms such as bacteria, fungi and insects (6). Different plant parts have shown biological activities viz., antipyretic, anti-

inflammatory, wound healing, analgesic, antidiarrhoeal, antioxidant and as an anti-diabetic, antinociceptive, fibrinogenolytic, anti-coagulant. Latex has good ovicidal and larvicidal properties etc. The prevalence of invasive, opportunistic microbial and fungal infections has increased at an alarming rate especially in immune-compromised individuals. Although it appears to be a great array of antimicrobial and antifungal drugs, there is at present a quest for new generations of antimicrobial and antifungal compounds due to the low efficacy, side effects or resistance associated to the existing drugs (7). This plant has potential antimicrobial properties against microbial infections (8-10).

The plant extracts in different solvents were screened for the presence of various bioactive phytochemicals. The analysis revealed the presence of cardic glycosides, saponins, flavonoids, steroids and terpenoids in most prominent amount while alkaloids and tannins in less amount. Anthraquinone is not present in none of the extracts in various solvents viz. n-hexane, ethanol, methanol, water, chloroform and ethyl acetate of *C.gigantea* (11).

C.gigantea has the following potential pharmacological properties; wound healing (12), antidiarrhoeal (13), CNS depressant activity (14), antipyretic and analgesic (15) anti-inflammatory (16), analgesic activity (17). The alcohol extract of the flower of *C.gigantea* reported analgesic activity in chemical and thermal models in mice. The roots of *C.gigantea* have been used in leprosy, eczema, syphilis, elephantiasis, ulceration, and cough in the Indian system of traditional medicine. It contains alkaloids, tannins, phenols and resins (17). Tetra and pentacyclic triterpenoids, cardiac glycosides. Its use in hepatitis has been illustrated in Indian System of Medicine. The folks and Vaidyas have clinically used it successfully. But no studies have been reported on phytochemical analysis and Antidiabetic activity. This motivated the biological evaluation of antidiabetic activity of crude plant extracts.

2.0 MATERIALS AND METHODS

2.1 Collection of Plant Material: The fresh leaves, flowers and stem of plant *C.gigantea* collected from the campus of Mohamed sathak college, Chennai, India. The leaves, flowers and stem were cut into small pieces and shade dried at room temperature, dried leaves, flowers and stem was subjected to size reduction to a coarse powder by using a dry grinder.

2.2 Preparation of Extract: 5g of finely ground Plant sample (leaves, flowers and stem) was extracted with 50ml of acetone, benzene, ethanol, methanol, hexane, ethyl acetate, diethyl ether and chloroform. The extracted residues were weighed and re dissolved in different solvents to yield 10mg/ml solutions. The crude extract thus obtained was stored in an air-tight container and used for further analysis. The organic extracts were further was used for analysis of phytochemicals, GC-MS analysis, antioxidant, antidiabetic, activity of *C.gigantea* (18).

2.3 Qualitative Analysis on Phytochemical Constituents: Qualitative phytochemical analysis were conducted using plant extract following standard methods (19).

2.4 Quantitative Analysis

2.4.1. Determination of Total Phenolic Content: The total phenolic content (TPC) of the crude extracts of leaves, flowers and stem were determined using the standard method with slight modifications (20). To 0.5ml of test sample, 1.5ml (1:10 v/v diluted with distilled water) Folin-Ciocalteau reagent was added and allowed to stand for 5min at 22ºC. After 5min, 2.0ml of 7.5% of sodium carbonate was added. These mixtures were incubated for 90min in the dark with intermittent shaking. After incubation the development of blue colour was measured at 725nm using a UV-Visible spectrophotometer. The phenolic content was calculated as gallic acid equivalents GAE/g on the basis of standard curve of gallic acid. The results were expressed as Gallic acid equivalents (GAE)/g of the plant material. All the determinations were carried out three times.

2.4.2. Determination of Total Flavonoid Content: The total flavonoid content (TFC) of different parts such as leaves, flowers and stem of *C.gigantea* was determined using the aluminium chloride assay through colorimetry (21). An aliquot (0.5 ml) of extracts were taken in different test tubes then 2ml of distilled water was added followed by the addition of 0.15ml of sodium nitrite (5% $NaNO_2$, w/v) and allowed to stand for 6min. Later 0.15ml of aluminium trichloride (10% $AlCl_3$) was added and incubated for 6min, followed by the addition of 2 ml of sodium hydroxide (NaOH, 4% w/v) and volume was made up to 5ml with distilled water. After 15min of incubation the mixture turns to pink, the absorbance was measured at 510nm using a colorimeter. Distilled water was used as blank. The TFC was expressed in mg of catechin equivalents (CE) per gram of extract. All the determinations were carried out three times.

2.4.3. Determination of Total Tannin Content: The tannins were determined by Folin and Ciocalteu method (22). 0.1ml of the sample extract was added with 7.5ml of distilled water and added 0.5ml of Folin Phenol reagent, 1ml of 35% sodium carbonate solution and diluted to 10ml with distilled water. The mixture was shaken well, kept at room temperature for 30min and absorbance was measured at 725nm using a UV-Visible spectrophotometer. Blank was prepared with water instead of the sample. A set of standard solutions of gallic acid is treated in the same manner as described earlier and read against a blank. The results of tannins are expressed in terms of gallic acid mg/g of extract.

2.5 GC-MS Identification of Bioactive Constituents: GC-MS analysis of the ethanol extract of *C.gigantea* leaf, flower, stem was performed using a Perkin Elmer GC Claurus 500 system and Gas Chromatograph interfaced to a Mass Spectrometer equipped with a Elite 5MS fused silica capillary column (30 × 0.25 mm ID. ×1 Mm df, composed of 5% Diphenyl/ 95% Dimethyl poly siloxane). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1ml/min and an injection volume of 3 Ml was employed (split ratio of 10:1). Injector temperature 250ºC; Ion-source temperature 280ºC. The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10 ºC/min to 200°C, then 5ºC/min to 280ºC ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV, a scan interval of 0.5 seconds and fragments from 45 to 450 Da. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Turbo Mass Ver 5.2.0. Interpretation on mass-spectrum GC-MS was conducted using the database of National institute Standard and Tecnology (NIST) having more 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library and the molecular weight and structure of the components of the test materials were ascertained.

2.6 Determination of Antioxidant Activities

2.6.1 Estimation of Radical Scavenging Activity (RSA): The RSA activity of different extracts were determined using DPPH assay according to standard method (23). The decrease of the absorption at 517nm of the DPPH solution after addition of the antioxidant was measured in a cuvette containing 2960µl of 0.1mM ethanolic DPPH solution was mixed with 40µl of 20 -

200µg/ml of *C.gigantea* extract. Blank containing 0.1mM ethanolic DPPH solution without *C.gigantea* extract and vortexed thoroughly, the setup was left at dark at room temperature. The absorption was monitored after 20min. Ascorbic acid (AA) and Butylated hydroxytoluene (BHT) were used as references. α tocopherol was used as a standard. The ability to scavenge DPPH radical was calculated by the following equation.

$$\text{\% of DPPH radical scavenging activity (\% RSA)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where,

$A_{control}$ is the absorbance of DPPH radical + ethanol

A_{sample} is the absorbance of DPPH radical + extract.

Measurements were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve vs percentage of inhibition.

2.7 *In vitro* Antioxidant Activity

2.7.1 Thiobarbituric acid (TBA) Method: Two ml of 20% trichloroacetic acid and 2ml of 0.67% 2-thiobarbituric acid was added to 1ml of sample solution, as prepared in FTC method. The mixture was placed in a boiling water bath, after cooling, centrifuged at 3000 rpm for 20min. Absorbance of supernatant was measured at 552nm. α tocopherol was used as a standard (24).

2.7.2 Superoxide Anion Radical Scavenging Assay: 1ml of NBT (Nitro blue tetrazolium) solution (156µM NBT in 100mM phosphate buffer, pH 8) mixed with 1ml of NADH (Nicotinamide adenine dinucleotide) solution (468µM in 100mM phosphate buffer, pH 8). Then the solution was mixed with 0.1ml of sample solution (10mg/ml). The reaction was started by adding 100µl of PMS (Phenazine methosulfate) solution (60µM PMS in 10mM, Phosphate buffer, Ph 8). The mixture was incubated at 25ºC for 5minutes. A control was performed with reagent mixture but without the sample. Absorbance was measured spectrophotometrically at 560 nm. α tocopherol was used as a standard (25).

2.7.3 Metal Chelating Activity: The chelating of ferrous ions by *C.gigantea* extract was estimated. The extract samples (250µl) were added to a solution of 2mmol/l $FeCl_2$ (0.05ml). The reaction was initiated by the addition of 5mmol/l ferrozine (0.2ml) and the mixture was shaken vigorously and left standing at room temperature for 10min. Absorbance of the solution was then measured spectrophotometrically at 562nm. The chelating activity of the extracts was evaluated using EDTA as standard (26).

2.7.4 Phospho molybdenum Assay: The antioxidant activity of samples was evaluated by the green phospho molybdenum complex formation according to the standard method (27). An aliquot of 100µl of sample solution was combined with 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) in a 4ml vial. The vials were capped and incubated in a water bath at 95°C for 90min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695nm against a blank.

2.7.5 Catalase: The reaction mixture contained 1.0ml of 0.01M phosphate buffer (pH 7.0), 0.1ml 1:10 diluted extract and 0.5ml of 2M hydrogen peroxide. The reaction was stopped by the addition of 2.0ml of dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). The tubes were heated in a boiling water bath for 10min. After cooling the contents, the optical density was measured at 590nm. The activity was expressed as units/mg protein. One unit of enzyme activity was defined as the amount of enzyme required to decrease the absorbance by 0.5 units at 590nm (28, 29).

2.7.6 Superoxide dismutase: The SOD activity was set up with an incubation medium in a final volume 3.0ml, containing equal volume of 50mM potassium phosphate buffer pH 7.8, 45µM methionine, 5.3mM riboflavin, 84µM NBT and 20µM potassium cyanide and 0.5ml of extract. The tubes were placed in an aluminium foil lined box maintained at 25°C and equipped with 15W fluorescent lamp and the reduced NBT was measured spectrophotometrically at 600nm after 10 minutes exposure to light. The maximum reduction was evaluated in the absence of enzyme. The activity was expressed in units/mg protein. One unit of enzyme activity was defined as the amount of enzyme that gives 50 percent inhibition of the extent of NBT reduction (30,29).

2.7.7 Glutathione Peroxidase: Glutathione peroxidase activity was carried out with a reaction mixture containing 0.4ml of sodium phosphate buffer (0.4M, pH 7.0); 0.1ml of 10mM sodium

azide, 0.2ml of 4mM reduced glutathione, 0.5ml of extract, 0.5ml of 2.5mM hydrogen peroxide and the final volume was made up to 2.0ml. The tubes were incubated at 37°C for 3 minutes. The reaction was terminated by the addition of 0.5ml of 10% trichloro acetic acid. To determine the residual glutathione the supernatant was removed by centrifugation. To this 3.0ml of 0.3M disodium hydrogen phosphate and 1.0ml of Dithio nitro benzoic acid (DTNB) were added. The colour developed was read at 412nm. The GPx activity was calculated and expressed as units/mg protein. One unit of GPx activity was defined as the µg of glutathione utilized/mg protein (31, 29).

2.7.8 Reduced Glutathione: The reduced glutathione was measured by its reaction with DTNB, for the GSH determination in the tissues (0.5ml) by precipitating the proteins by adding 0.12ml of 25% trichloro aceticacid, the precipitated proteins were centrifuged at 1000Xg for 10 minutes. The supernatant was cooled on ice and 0.1ml of the supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0) and 0.2ml of freshly prepared DTNB (0.6mM) was added to the tubes and the intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10 minutes. The results were expressed as nmols/g extract (32).

2.8 Determination of Antidiabetic activities

2.8.1 Cell line and culture: *RIN-5F* cell lines were obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in Minimal Essential Medium supplemented with 10% FBS (Fetal Bovine Serum), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50µg/ml CO_2 at 37°C. MEM was purchased from Hi Media Laboratories Fetal Bovine Serum (FBS) was purchased from Cistron laboratories Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

2.8.2 *In Vitro* assay for Antidiabetic activity (MTT assay): RIN-5F Pancreatic cells (1×10^5/well) were plated in 24 well plates and incubated in 37^0C with 5% CO_2 condition. After the cell reaches the confluence, the samples were added and incubated for 24 hours. After incubation, the sample was removed from the well and washed with phosphate-buffered saline

(pH 7.4) or MEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The % cell viability was calculated using the following formula:

$$\% \text{ cell viability} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

Where,

A_{570} is the absorbance at 570nm (33).

3.0 RESULTS

3.1 Qualitative analysis of phytochemical constituents of *C.gigantea*

Direct extraction with different solvent was used for the purpose of preliminary screening of *C.gigantea.* The preliminary phytochemical screening of *C.gigantea* extract revealed the presence of carbohydrates, proteins, amino acid, saponin, tannin, flavanoids, alkaloids, glycosides and polyphenol and absence of carotenoid **(Table 1).**

TABLE 1. Phytochemical screening of *C.gigantea*

S.No.	Compound	Acetone			Benzene			Ethanol			Methanol			Hexane			Ethyl acetate			Diethyl ether			Chloroform		
		L	F	S	L	F	S	L	F	S	L	F	S	L	F	S	L	F	S	L	F	S	L	F	S
1.	Carbohydrates	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2.	Fatty acids	-	+	+	-	+	-	-	+	+	-	+	+	-	-	-	-	+	+	-	+	-	-	-	-
3.	Proteins	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	+
4.	Amino Acids	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
5.	Saponins	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+
6.	Tannins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.	Carotenoids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

8.	Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
9.	Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
10.	Glycosides	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11.	Poly phenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

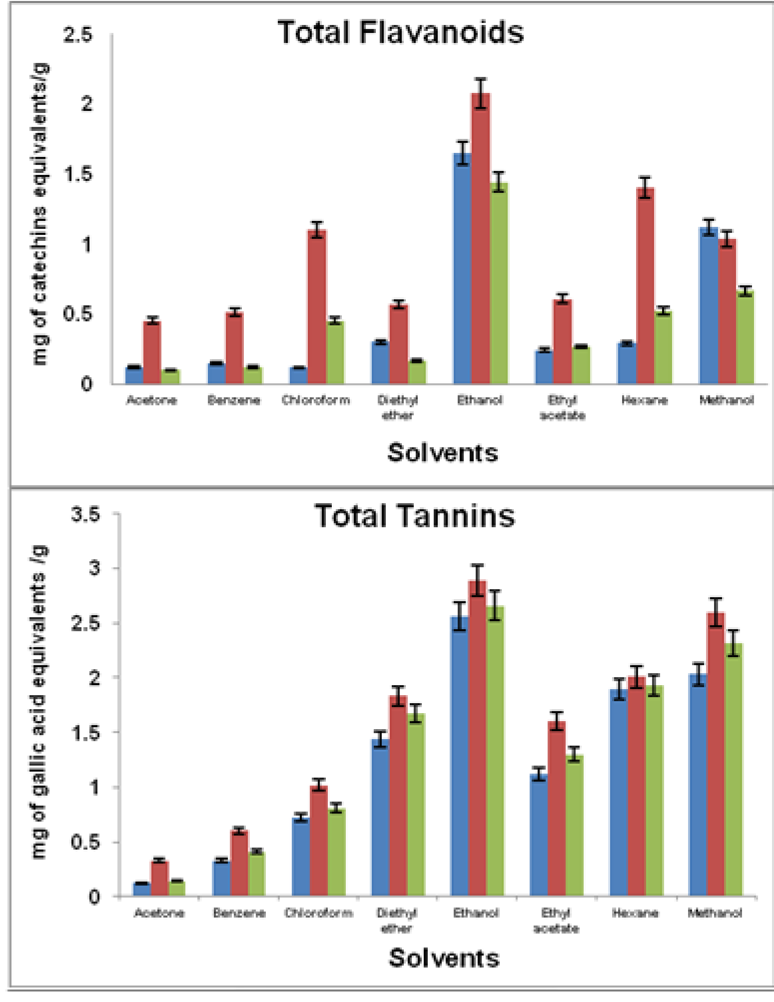
The values are analysis of triplicate.

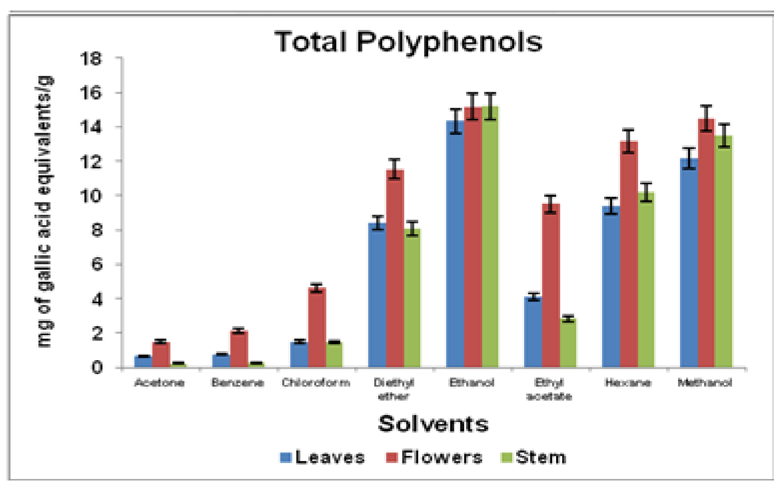
L - Leaves, F – Flowers, S – Stem

+ Present - Absent

3.2 Quantitative Analysis of phytochemicals from *C.gigantea*

In the present study the phenol, flavonoid and tannin content of *C.gigantea* flower extract was found to be 15.16±0.068, 2.075, 2.89 mg gallic acid equivalent (GAE), per gram plant extract **(Figure 1).**





Values are mean±SD of triplicate.

FIGURE 1. Quantitative analysis Phytochemicals from *C.gigantea*

3.3. GC-MS identification of bioactive constituents

The ethanol extract of *C.gigantea* flower contains rich phytochemical constituents which in turn resulted in the identification of thirty six different compounds by GC/MS analysis. The prevailing bioactive components with their Retention time (RT), Molecular formula, Molecular weight (MW) and concentration (%) are presented in **Figure 2, Table 2**.

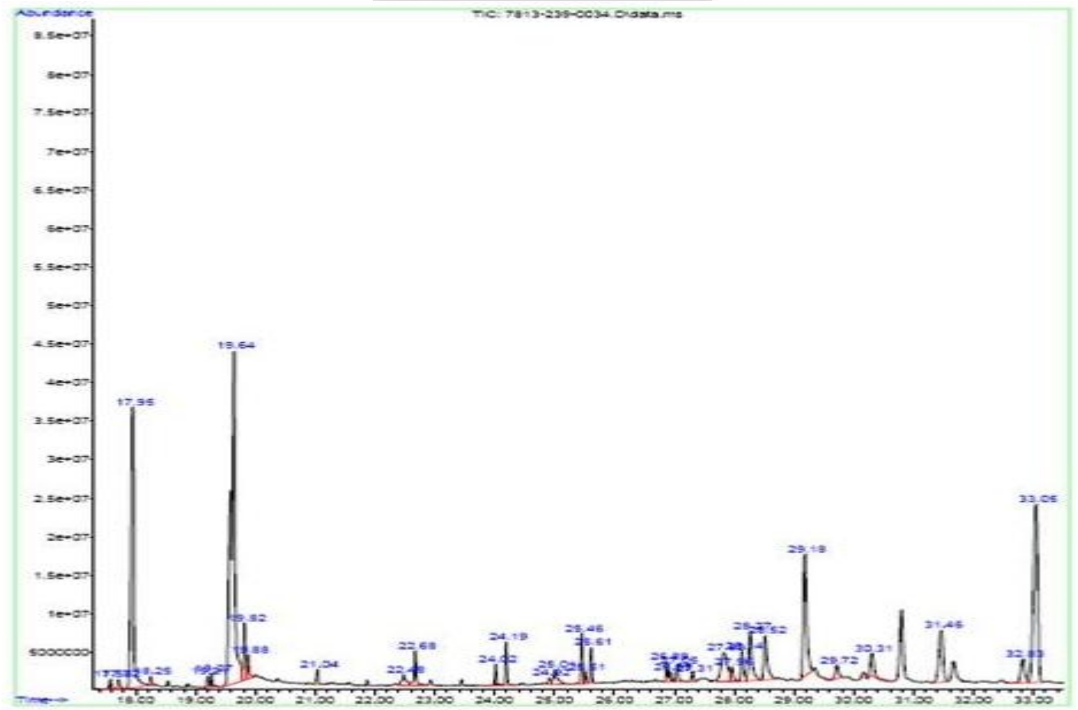


FIGURE 2. GC-MS Analysis of Ethanolic extracts *C.gigantea* flowers

TABLE 2. GC-MS Identification of Bioactive constituents in ethanol extract of *C.gigantea flowers*

S.No.	Retention Time	Peak Area %	Name of the Compound	Molecular Formula	Molecular Weight (g mol^{-1})
1	6.06	0.55	2-Propanone, diethylhydrazone 2-Aminomethyl-5-methylamino-1,3,4- oxadiazole Furan, 2-methyl-5-(methylthio)-	$C_6H_{14}N_2$	114.19
2	10.35	1.69	2-Methoxy-4-vinylphenol Benzene,[2-(methylthio)ethenyl]-(Z)- 2,4,6-Trimethyl-1,3-phenylenediamine	$C_9H_{10}O_2$	150.1745
3	17.58	0.31	Hexadecanoic acid, methyl ester Hexadecanoic acid, methyl ester Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507
4	17.71	0.43	cis-9-Hexadecenoic acid Palmitoleic acid Z-7-Hexadecenoic acid	$C_{18}H_{34}O_2$	282.46
5	17.96	15.03	cis-9-Hexadecenoic acid Palmitoleic acid Z-7-Hexadecenoic acid n-Hexadecanoic acid n-Hexadecanoic acid Tetradecanoic acid	$C_{17}H_{32}O_2$	268.4348
6	18.25	0.29	Hexadecanoic acid, ethyl ester Hexadecanoic acid, ethyl ester Tetradecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284.4772
7	19.21	0.34	9,12-Octadecadienoic acid (Z,Z)-, methyl ester Methyl 10-trans,12-cis-octadecadie noate 9,15 Octadecadienoic acid, methyl ester, (Z,Z)-	$C_{18}H_{32}O_2$	280.4454 8
8	19.27	0.40	9-Octadecenoic acid, methyl ester, (E)-9-Octadecenoic acid (Z)-, methyl ester trans-13-Octadecenoic acid, methyl	$C_{19}H_{36}O_2$	296.4879
9	19.64	25.01	Oleic Acid 9-Octadecenoic acid, (E)-cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.46

10	19.82	1.90	Octadecanoic acid Octadecanoic acid 9,17-Octadecadienal, (Z)-	$C_{18}H_{36}O_2$	284.4772 03
11	19.88	0.90	9,12-Octadecadienoic acid (Z,Z)- 9,17-Octadecadienal, (Z)- 2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{28}O_2$	252.3923 20
12	21.04	0.51	Eicosane Nonadecane, 9-methyl- Heptadecane	$C_{20}H_{42}$	282.55
13	22.49	0.87	Pyrene, hexadecahydro-.beta.-Amyrin4,4,6a,6b,8a,11,11,14b-Octamethyl1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	$C_{16}H_{10}$	202.25
14	22.68	1.48	Nonadecane Nonadecane, 9-methyl- Hexacosane	$C_{26}H_{54}$	366.71
15	24.01	0.67	Z-5-Nonadecene Behenic alcohol Dichloroacetic acid, heptadecylester	$C_{19}H_{38}$	266.51
16	24.19	1.20	Nonadecane, 9-methyl- Hexacosane Nonadecane	$C_{19}H_{40}$	268.53
17	24.91	0.30	Eicosane Eicosane Nonadecane,	$C_{20}H_{42}$	282.55
18	25.02	1.13	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-5(1H)-Azulenone, 2,4,6,7,8,8a-hexa hydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)-Indeno[2,1-b]chromene,	$C_{27}H_{42}O_5$	446.62
19	25.47	1.40	1-Docosene 1-Heptacosanol Heptacosyl acetate	$C_{27}H_{56}O$	19.585
20	25.51	0.38	1-Heptacosanol Octacosyl acetate n-Tetracosanol-1	$C_{28}H_{58}O$	410.76
21	25.61	1.16	Eicosane Nonadecane Heptadecane	$C_{22}H_{46}$	310.6006 40
22	26.89	0.72	1-Heptacosanol 1-Nonadecene n-Tetracosanol-1	$C_{19}H_{38}$	20.395

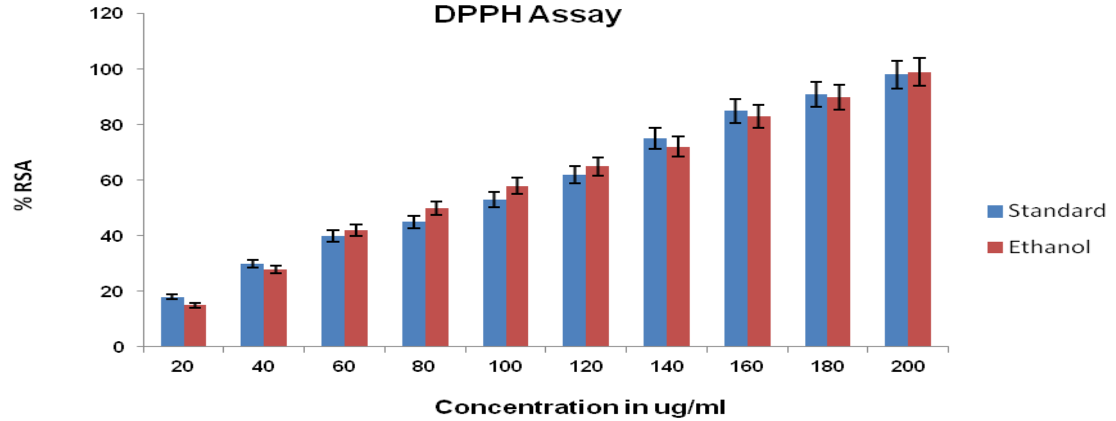
23	26.95	0.38	Heneicosyl trifluoroacetate Octacosanol 1-Nonadecene	$C_{18}H_{31}O_2F_3$	336.55
24	27.05	0.70	Eicosane Heptadecane Nonadecane	$C_{20}H_{42}$	254.49
25	27.31	0.41	Vitamin E dl-.alpha.-Tocopherol Vitamin E	$C_{31}H_{52}O_3$	472.74278
26	27.83	3.40	Toluene, 4-chloro-2-fluoro-5-nitro Cobalt, .[(1,2,3,4-.eta.)-1,3-cycl opentadiene](.eta.5-2,4-cyclopentadien-1-yl)-Ethanone, 1-[4-(trifluoromethoxy)phenyl]-	$C_7H_6C_lF$	144.57
27	27.96	0.79	Phenol 3-Ethoxy-4-methoxybenzaldehyde 1H-Isoindole-1,3(2H)-dione, 2-buty l-4,5,6,7-tetrahydro-	$C_8H_{10}BFO_3$	183.972603
28	28.14	1.43	Butyldimethylsilyloxybenzene .gamma.-Tocopherol .gamma.-Tocopherol	$C_{29}H_{50}O_2$	430.71
29	28.27	3.32	Campesterol 5-Cholestene-3-ol, 24-methyl- Campesterol	$C_{28}H_{48}O$	400.6801
30	28.52	2.84	Stigmasterol Stigmasterol Stigmasterol	$C_{29}H_{48}O$	412.69
31	29.18	7.72	.gamma.-Sitosterol .beta.-Sitosterol .beta.-Sitosterol	$C_{29}H_{50}O$	414.7067
32	29.72	0.80	.beta.-Amyrin .alpha.-Amyrin 4,4,6a,6b,8a,11,11,14b-Octamethyl1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	$C_{30}H_{50}O$	426.73
33	30.30	1.05	.alpha.-Amyrin .alpha.-Amyrin .alpha.-Amyrin	$C_{30}H_{50}O$	426.73
34	31.46	4.13	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	$C_{31}H_{48}O_3$	468.7110

			.alpha.-Amyrin 12-Oleanen-3-yl acetate, (3.alpha.)-		2
35	32.83	1.87	Urs-20-en-3-ol, (3.beta.,18.alpha.,19.alpha.)- A'-Neogammacer-22(29)-en-3-ol, acetate, (3.beta.,21.beta.)- Cobalt, .[(1,2,3,4-.eta.)-1,3-cyclopentadiene](.eta.5-2,4-cyclopentadien-1-yl)-	$C_{30}H_{50}$	426.7174
36	33.05	14.53	A'-Neogammacer-22(29)-en-3-ol, acetate, (3.beta.,21.beta.)-14-Nor-3-acetoxyurs-12-ene, 14.alpha.,20.beta.-Lup-20(29)-en-3-ol, acetate, (3.beta.)-	$C_{30}H_{50}$	410.72

3.4. Antioxidant activities

3.4.1. Radical scavenging activity of *C.gigantea* extracts

From the dose dependent response curve of DPPH radical scavenging activity of *C.gigantea* flowers was observed that had higher radical scavenging activity. At a concentration of 80µg/ml of ethanol extract shows, the scavenging activity of 50%, which was compared to that of standard ascorbic acid (74%) compared with other organic extract. The ethanol extract of *C.gigantea* flower showed excellent antioxidant and free radical scavenging activity when compared with acetone, benzene, methanol, ethyl acetate, diethyl ether, chloroform and hexane. In considering this, the ethanol extract of flower was chosen for further study. The values obtained were plotted in graph. **(Figure 3)**

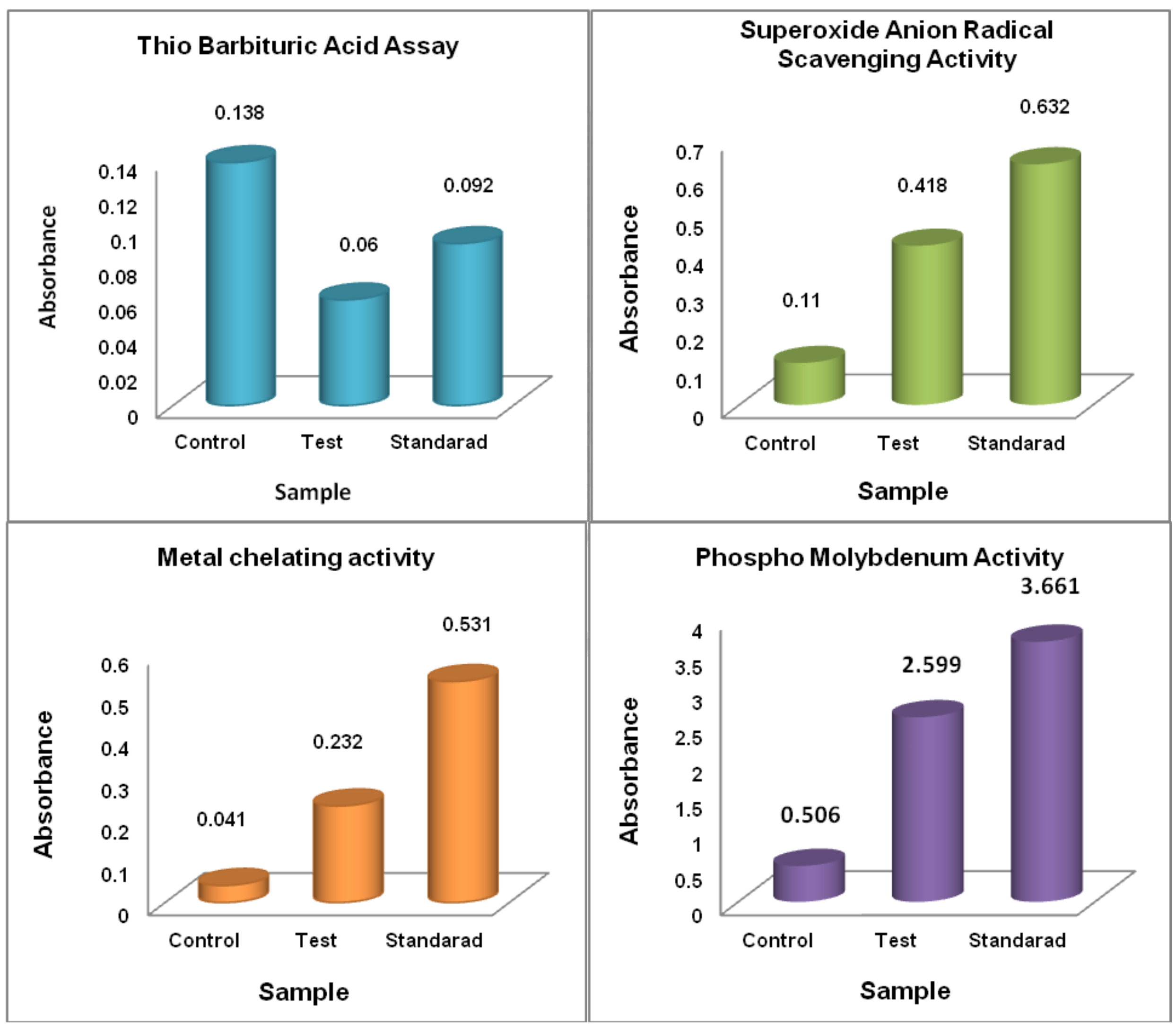


Values are mean of ± SD of triplicates.

FIGURE 3. Radical scavenging activity (RSA) ethanol extract of *C.gigantea* leaves

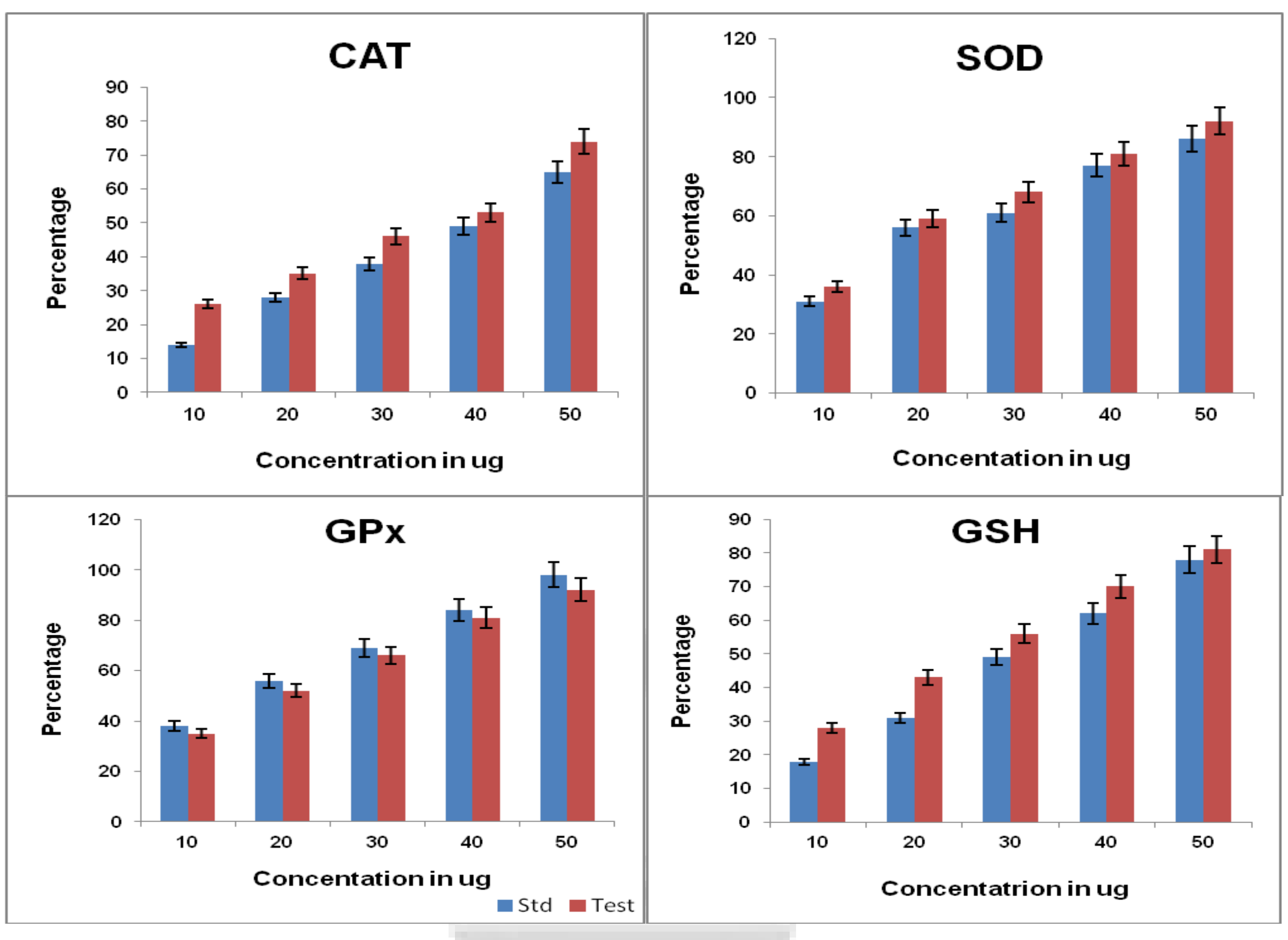
3.4.2. *In Vitro* Antioxidant Activities of *C.gigantea* flower Extract

In vitro antioxidant activity of the selected medicinal plant flowers were tested by various methods includes thio barbituric acid assay, superoxide anion scavenging assay, metal chelating activity, Phospho molybdenum activity. These assays showed excellent antioxidant activity exhibited by the *C.gigantea* flowers are shown in **Figure 4**. The activity of catalase, superoxide dismutase, Glutathione peroxidase and Reduced glutathione *C.gigantea* flower extract was tested and the results obtained are indicated in **Figure 5**.



Values are mean of triplicates.

FIGURE 4. *In Vitro* Antioxidant Activities of *C.gigantea* flower extract



Values are mean of ± SD of triplicates.

FIGURE 5. *In Vitro* Antioxidant Activities of *C.gigantea* flower extract

3.5. Antidiabetic activity of *C.gigantea*

The result of preliminary qualitative, quantitative and GC-MS analysis confirms the flower ethanol extract contains more bioactive compounds with antioxidant activity. So, the flower ethanol extract was further used for the analysis of antidiabetic potentials using pancreatic RIN-5F cell line. The Antidiabetic activity of the Ethanol extract of *C.gigantea* flowers was subjected for MTT assay. In this assay cell death and cell viability of pancreatic cell line of antidiabetic activity was estimated. The results showed 10.71% cell viability in the concentration of 1 mg/ml.

The IC_{50} of cell viability was observed at concentration of 62.5 µg/ml of the ethanol extract as 50% .The values obtained were plotted in graph **(Figure 6, Figure 7)**.

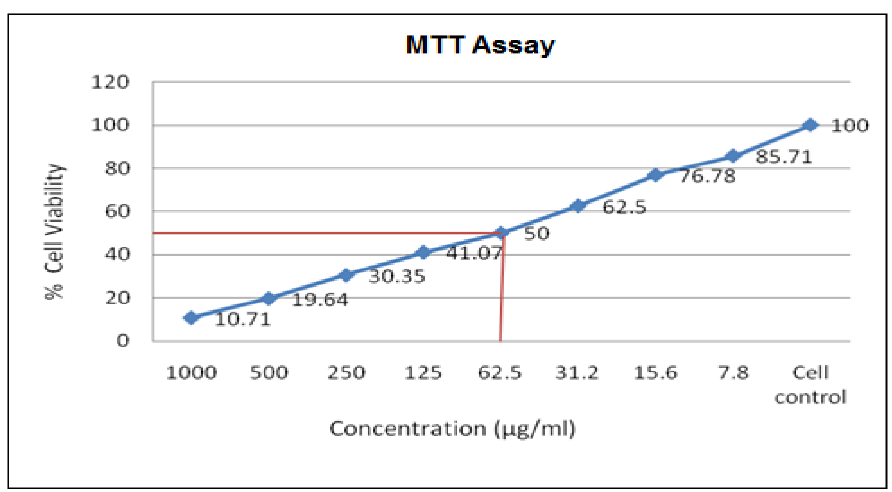


FIGURE 6. Antidiabetic activity of *C.gigantea* in pancreatic RIN-5F cell line

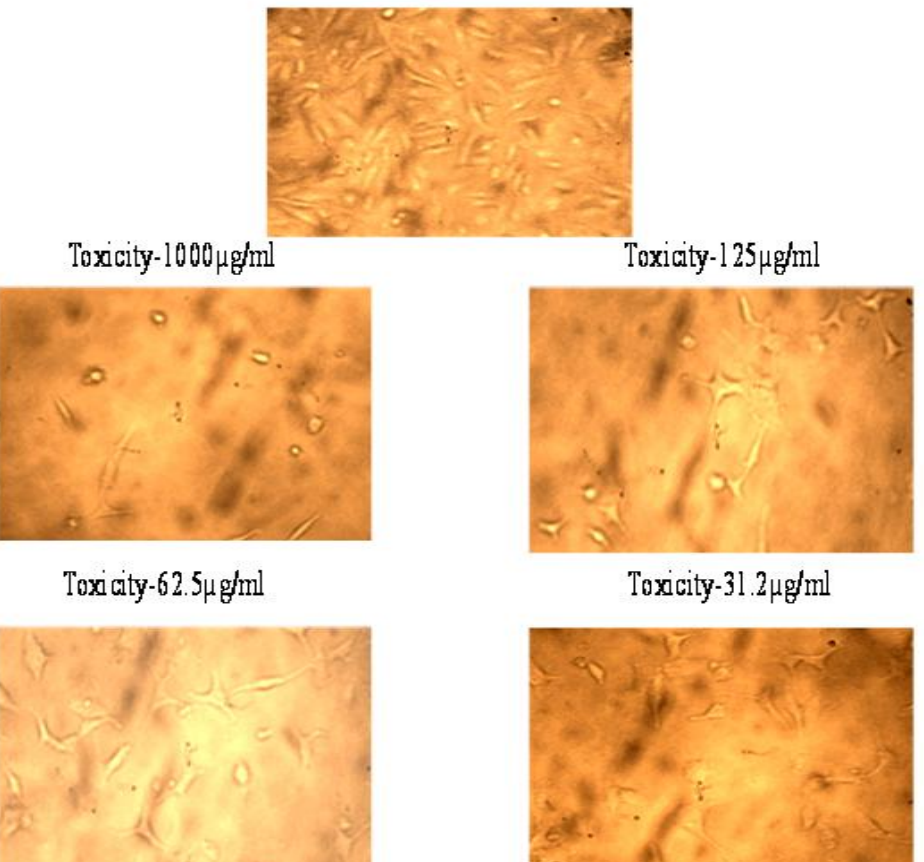


FIGURE 7

4.0 DISCUSSION

Qualitative analysis was carried out on *C.gigantea* showed the presence of phytochemical constituents. It shows that carbohydrates, proteins, amino acid, saponin, tannin, flavanoids, alkaloids, glycosides and polyphenol were present. Looking for the previous studies, the presence of these phytochemical compounds is known to support the bioactivities of medicinal plants (34-35) and thus it was very important elements responsible to evaluate antioxidant activities for *Calotropis* extract in this study.

Plant polyphenol, flavonoid and tannin are a major group of compounds which have the following effects; choleretic and diuretic functions, decreasing blood pressure, reducing the viscosity of the blood and stimulating intestinal peristalsis, as well as primary antioxidation or free radicals scavenging activities (35).

The most important phytochemicals in plant are phenolics whereas there are more than 8000 phenolic phytochemicals (36). These phenolic compounds interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. Moreover, their bioactivities may be related to their ability to inhibit lipoxygenase and scavenge free radicals (37). Probably the most important natural phenolics are flavonoids and tannin which contain hydroxyl functional groups, because of their broad spectrum of chemical and biological activities, responsible for antioxidant effect of the plants. So, the true antioxidant potential is often more accurately revealed by expressing antioxidant activity in terms of phenolics, flavonoids and tannin content. Therefore, in this study, the obtained level of phenolics, flavonoid and tannin in *C.gigantea* extract may be a sign to suggest that the extract has antioxidant activity. Our suggestion is in close agreement with previous reports that there is a strong correlation between the total phenolic, flavonoids and tannin content and antioxidant activity of extract from plant (38, 26).

The radical scavenging activity of *C.gigantea* shows the capacity of the plant extract to scavenge the free radical DPPH. The IC_{50} of radical scavenging activity was observed at concentration of 80µg/ml of the extract as 51% which was compared to the standard α-tocopherol (76%).

The ethanol extract of *C.gigantea* flowers have strong superoxide radical scavenging activity and exhibited higher superoxide radical scavenging activity than control. Superoxide anions indirectly initiated lipid oxidation as a result of superoxide and hydrogen peroxide serving as

precursors of singlet oxygen and hydroxyl radicals. Superoxide anion radical ($O2^{\cdot -}$) is a weak oxidant but it gives rise to the generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both free radicals contribute to oxidative stress (39).

Presence of transition metal ions in a biological system could catalyse the Haber–Weiss and Fenton-type reactions, resulting in generation of hydroxyl radicals ($OH^{\bullet}$). However, these transition metal ions could form chelates with the antioxidants, which result in the suppression of $OH^{\bullet}$ generation, and inhibit ion of peroxidation processes of biological molecules. In this assay, the presence of chelating agents in the ethanol extract of *C.gigantea* flower disrupts the ferrozine–Fe^{2+} complex formation, thus decreasing the red color. The metal ion scavenging effects of *C.gigantea* is reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. The data presented in this study indicated that *C.gigantea* ability for iron binding and could reduce the generation of hydroxyl radicals. In the metal chelating activity, Ferrozine can quantitatively chelate with Fe^{2+} and form a complex with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{2+} complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (40).

The phospho molybdenum assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. The phospho molybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The different extract of *C.gigantea* leaves, flowers and stem were used to determine their antioxidant capacities by the formation of green phosphomolybdenum complex. The results indicate that the ethanol extract of *C.gigantea* flowers has more powerful antioxidant in the reduction of phosphomolybdenum complex.

Phospho molybdenum reduction potential of *C.gigantea* was explained by the fact that the transfer of electrons/hydrogen from antioxidants occurs at different redox potential in various assay system and the transfer also depends on the structure of the antioxidants (41).

The CAT activity at 50µg ml^{-1} concentration was found to be 63% that of BHA (Butylated hydroxyanisole) standard was found to be 72% at the concentration 50µg /ml. *C.gigantea* extract

was capable of scavenging superoxide dismutase in a concentration dependent manner. The extract significantly scavenged up to 58 % superoxide radicals at a concentration of 20μg/ ml which is lesser than that of BHA (60 %) at a concentration of 20μg/ml. The extract of *C.gigantea* was capable of scavenging glutathione peroxidase activity at a concentration of 20μg/ml was found to be 53 % which is lesser than that of BHA (58%) at a concentration of 20μg/ml. Glutathione reductase was significant increase activity compared to standard. *C.gigantea* extract at 30μg/ml concentration was found to be 50% that of BHA standard was found to be 30% at the concentration 57μg/ml.

C.gigantea extract was capable of scavenging free radicals through their catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase in a concentration dependent manner. It is known that free radical cause auto-oxidation of unsaturated lipids in food (42). On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation of lipid. Superoxide anion radical is not only one of the strongest reactive oxygen species among the generated free radicals but also a precursor to other active free radicals such as catalase, glutathione peroxidase, glutathione reductase, hydrogen peroxide, hydroxyl radical, and singlet oxygen, which play an important role in the oxidative damage in lipids, proteins, and DNA and thereby inducing tissue damage (43). It has been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical *in vitro* (44). Also, *in vivo*, the up to date study suggested that the flavonoids may involve the dismutation of superoxide anion radical. Our results showed that *C.gigantea* extract inhibited gradually, in a concentration dependent manner. This scavenging activity of the extract was comparable to that of standard antioxidant BHA suggesting that *C.gigantea* is a potent scavenger of superoxide .The findings of the present study suggested that the flower extract from this plant could be a potential natural source of antioxidants and could have greater importance as therapeutic agent in preventing or slowing diabetic activity. Phenolic compounds, including flavonoids, tannin are especially promising candidates for diabetic prevention.

4.0 CONCLUSION

In this study, the antidiabetic, and antioxidant activity of the medicinal plant of *C.gigantea* was carried out. Gas chromatography and mass spectroscopy analysis showed the existence of various compounds differ in their chemical nature. These compounds may possess good antidiabetic and leading to antioxidant activities. The antioxidant phytochemicals protect the cells from free radical attack and oxidative damage. Phenolic compounds, including flavonoids, tannin are especially promising candidates for diabetic prevention. Thus, consuming a diet rich in antioxidant plant foods (e.g. fruits and vegetables) will provide health-protective effects, lead us to propose *C.gigantea* as promising natural sources of antioxidants suitable for application in nutritional/pharmaceutical fields, in the prevention of free radical mediated diseases. The need for development of new antidiabetic drugs and more importantly from natural sources cannot be overemphasized *C.gigantea* provides a good opportunity for drug development in this area. In the present work, the high antioxidant and antidiabetic capacity observed for ethanol extract from *C.gigantea* flower and also it has a potential of preventing human diseases in which free radicals are involved, such as diabetes.

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Conflict of interest

The authors do not have any conflict of interest.

REFERENCES

1. Subbulakshmi G., Naik M. Indigenous foods in the treatment of diabetes mellitus. *Bombay Hospital Res J*, 2001; 43: 548-61.
2. Grover J.K., Yadav S., Vats V. Medicinal plants of India with anti-diabetic potential. *J. Ethnopharmacol*, 2002; 81: 81-100.
3. Arts I.C., Hollman P.C., Polyphenols and disease risk in epidemiologic studies. Am *J Clin Nutr*, 2005; 81(1): 317S-325S.
4. David G. Gardner, Dolores. Greenspan's basic & clinical endocrinology. New York: McGraw-Hill Medical, 2011,Chapter 17. ISBN 0-07-162243-8.
5. Watkins, John, V., Sheehan, thomas, J. and Black, Robert, J. Florida Landscape Plants: Native and Exotic. 2005. University Press of Florida, Gainesville, Florida.

6. Sharma A.K., Kharb R. and Kaur R. Pharmacognostical aspects of *Calotropis procera*. *Int J of Pharm and Bio Sci*. 2011; 2(3): 480-488.
7. Kanimozhi D, Ratha bai V, Baskaran C, Evaluation of Anti Microbial Activity of Acalypha indica. *Int J of Res in Pharm and Sci*. 2012; 2(1): 130-138.
8. Nenaah E.G., Mahmed M.E. Antimicrobial activity of extracts and latex of *Calotropis procera* and synergistic effect with reference antimicrobials. *Res J Medicinal plants*. 2011; 5(6): 706-716.
9. Mamta Goyal and Rashmi Mathur. Antimicrobial Potential and Phytochemical Analysis of Plant Extracts of Calotropis Procera. *Int J of drug discovery and herbal Res*. 2011; 1(3): 138-143.
10. Falguni K. Sheth, Minoo H. Parabia. Ethnobotanical studies and validation of lead: a case study on evaluation of *Calotropis sp*. on dermal fungal infections. *Int J of Pharm and Life Sci.*, 2011; 2(6): 797-800.
11. Sharma A.K., Kharb R., Kaur R. Pharmacognostical aspects of *Calotropis procera*. *Int J of Pharma and Bio Sci*. 2011; 2(3): 480-488.
12. Deshmukh P.T., Fernandes J., Atul A., Toppo E., Wound healing activity of *Calotropis gigantea* bark in rats, *J Ethnopharmacol*., 2009; 125: 178– 181.
13. Argal and A.K. Pathak. *Ind J Natural Products*, 2005; 21(4): 55-57.
14. Argal A.K., Pathak. Evaluation of Hepatoprotective Activity of *Calotropis gigantea* R.Br. Flowers, *Indian Journal of Applied Life Sciences*, 2006; 2: 41-43.
15. Bravo L., Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. *Nutr.Rev* , 1998; 56: 317-333.
16. Adak M, Gupta J.K., Evaluation of anti-inflammatory activity of Calotropis gigantea (AKANDA) in various biological system, *Nepal Med Coll J*., 2006; 3: 156–161.
17. H.R. Chitme, R. Chandra and S. Kaushik., Evaluation of analgesic activities of *Calotropis gigantea* extract *in vivo*. *Asia Pac J Pharmacol*., 2005b; 16: 35–40.
18. Evans, W.C., Pharmacognosy, 15th Edn., W.B. Saunders, New York, 2002; 471
19. Eloff, J.N. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica*, 1998; 64: 711–713.
20. Safowora A, Medical plats and Traditional medicine in Africa: Spectrum Books Ltd, Ibadan, Nigeria, 1993; 191-289.
21. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*., 1999; 299: 152-178.
22. Makkar, H.P.S., Blummel, M., Borowy, N.K. and Becker, K. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J Sci and Food Agri*, 1993; 61: 161–165.Nenadis N., Zafiropoulou I., Tsimidou M. Commonly used food antioxidants: Acomparative studyin dispersed systems. *Food Chem*, 2002; 82: 403 - 407.
23. Ottolenghi, A. Interaction of ascorbic acid and mitochondria lipids. *Arch. Biochem. Biophy.*, 1959; 79: 355-363.
24. Nishikimi, M., Rao, N.A., Yagi, K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem Biophys Res Commun,* 1992; 46: 849 - 854.
25. Pan Y., Wang, K., Huang, S. Antioxidant activity of microwave-assisted extract of longan (*Dimocarpus Longan Lour*.) peel. *Food Chem*, 2008; 106(3): 1264–1270.
26. Prieto, P., Pineda, M., Aguilar, M. Spectophotometric quantitative of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Anal Biochem*, 1999; 269: 337-341.
27. Sinha N, Baquer NZ, Sharma D, Anti lipidperoxidative role of exogenous dihydro epiendrosterone (DHEA) administration in normal aging at brain, *Ind J Exp Biol,* 1972; 43: 420-424.Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with the Foli-phenol reagent, *J Biol Chem*, 1951; 193(1): 265-275.
28. Misra HP, Fridovich I, Assay of superoxide dismutase, *J Biol Chem,* 1972; 247: 170-173.
29. Rotruck J.T., Pope A.L., Ganther H.E., Swanson A.B., Hafeman D.G., Hoekstra W.G., Selenium: Biochemical role as a component of glutathione peroxidase. *Sci*, 1973; 179: 588-590.

30. Moren M.S., Deperre J.W., Mannervik B., Levels of glutathione, glutathione reductase and glutathione transferase activities in rat lung and liver, *Biochem and Biophys Acta,* 1979; 582: 67-70.
31. Terry Sharrer, Hela Herself, *The Scientist,* 2006; 20: 22.
32. Jayaprakash G.K., Singh R.P., Sakariah K.K., Antioxidant activity of grape seed extracts on peroxidation models in vitro. *Agric. Food Chem.* 2001; 55: 1018 - 1022.
33. Pan Y., He C., Wang H., Ji X., Wang K. and Lui P. Antioxidant activity of microwave - assisted extract of Buddleia officinalis and its major active component. *Food chem.* 2010; 121: 497 - 502.
34. Kuti J.O. Antioxidant compounds from four Opuntia cactus pear fruit varieties, *Food Chem.* 2004; 85: 527 – 533.
35. Kessler M., Ubeaud G. and Jung L. Anti – oxidant and proactivity of rutin and quercetin derivatives. *Pharm Pharmacol,* 2003; 55: 131 - 142.
36. Cai Y.Z., Luo Q., Sun M. and Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci*, 2004; 74: 2157 – 2184.
37. Nenadis, N., Lazaridou, O. and Tsimidou, M.Z., Use of Reference Compounds in Antioxidant Activity Assessment. *J of Agri Food Chem*, 2007; 55: 5452-5460.
38. Elmastas, M., Gulcin, I., Beydemir, O.I., Kufrevioglu, H.Y., Aboul, E.H.Y. A study on the *in vitro* antioxidant activity of juniper (*Juniperus communis L.*) fruit extracts. *Anal Lett*, 2006; 39: 47.
39. Soler-Rivas C., Espin J.C., Wichers H.J. An easy and fast test to compare total free radical scavenger capacity of foodstuffs. *Phytochem Anal*, 2000; 11: 330–338.
40. Loo AY, Jain K, Darah I., Antioxidant activity of compounds isolated from the pyroligneous acid, *Rhizophora apiculata*. *Food Chem*, 2008; 107: 1151 - 1160.
41. Kaur G., Tirkey N., Bharrhan S., Chanana V., Rishi P. and Chopra K. Inhibition of oxidative stress and cytokine activity by curcumin in amelioration of endotoxin-induced experimental hepatotoxicity in rodents. *Clin Exp Immunol,* 2006; 145: 313–321.
42. Gulcin, I., Antioxidant and antiradical activities of L-carnitine. *Life Sci,* 2006; 78: 803–811.
43. Ak T., Gülçin L., Antioxidant and radical scavenging properties of curcumin., *Chem Biol Interact*. 2008; 174: 27–37.
44. Demir F., Uzun F.G., Durak, D. and Kalender Y. Subacute chlorpyrifos- induced oxidative stress in rat erythrocytes and the protective effects of catechin and quercetin. Pesticide, *Biochem Physiol*, 2011; 99: 77- 81.