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In vitro antidiabetic potential and GC-MS analysis of *Digera muricata* and *Amaranthy cruentus*

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Abstract
The present study focuses on two plants of the family *Amaranthaceae*, and order *Caryophyllales* viz., *Digera muricata* and *Amaranthy cruentus*. All the medicinal plants were biologically active and plays major role in curing various hazardous human diseases. Therefore, the present work was undertaken to explore phytochemical analysis of these samples from the leave tissues and finally after analysis these are considered as rich source of phytoconstituents. The leaves of the selected two medicinal plants were washed, air dried and then powdered. Totally five different extracts namely aqueous, methanol, ethanol, chloroform and acetone extract of leaf samples were used for the phytochemical analysis to compare the phytochemical constituents in the selected plants. The results of the phytochemical analysis of these medicinal plants showed that the alkaloid, flavanoids, glycoside, amino acids, fatty acids, carbohydrates, proteins, phenolioc, tannins, steroids, terpenoids, anthocyanins, leucoanthocyanins, coumarins and emodins were found to be present in afore mentioned medicinal plants. The GC chromatograms was also performed on methanol extract and showed the retention time in the column and detected the peaks which correspond to the bioactive compounds present in the extract. The presence of polyphenol and antioxidant constituents such as flavonoids from the selected medicinal plants showed to have the ability to reduce blood glucose levels. The present finding reveals that the methanol extract of *Digera muricata* effeciently reacts with alpha-amylase in an extract dependent manner than *Amaranthy cruentus* by *in-vitro* analysis. Hence, the present work was undertaken to evaluate and compare the antidiabetic potential and also to support of the traditional claims.

Keywords: Phytoconstituents, *Digera muricata*, *Amaranthy cruentus*, GC chromatograms and antidiabetic, α-amylase activity

Introduction
Both primary and secondary phytoconstituents are ubiquitously present in the roots, leaves, shoots, flowers, vegetables and fruits of the medicinal plants that naturally exhibits defense mechanism, pharmacological activities i.e., anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral, anti-bacterial and ant-diabetic activities (Abdul Wadood *et al*., 2013) [1]. Diabetes mellitus (DM) is a complex metabolic disease caused by improper insulin secretion by the pancreas leading to hyperglycaemia. The symptoms of this disease are not recognized that much easily by an individual unless a patient undergoes diagnosis. This makes the disease more vigorous and causes long term complications related to damage to blood vessels, cardiovascular diseases, tissue scarring, loss of urine protein, chronic kidney disorders etc. Therefore the presence of phytoconstituents in the medicinal plants play a major role by both contolling as well as by preventing the diseases by increasing the efficiency of the glucose metaboilism, lipid metabolism, antioxidant status, and capillary function. The current research has focused on two such medicinal plants from the same family *Amaranthaceace* namely, *Digera muricata*, and *Amaranthus cruentus*. All over the world, its estimated 2.85% of the population is affected by this disease and it is expected to increases upto 5.4% in the year of 2025 (Patel *et al.*, 2012) [9]. Several antidiabetic therapies like Sulphonylureas, Meglitinides, Biguanides, Thiazolidinediones, Alpha-glucosidases inhibitor, Dipeptidyl-peptidase-4-inhibitors, Glucagon-like peptide-1-agonist, Sodium glucose cotransporter-2-inhibitors etc. are regularly used (Saxena & Vikram, 2004) [12]. But usage of such drugs caused severe side effects like hypoglycemia, diarrhea, metallic aftertaste, nausea, pharyngitis, headache, vomiting, pioglitazone, rosiglitazone, genital and urinary infections. Therefore current research focus on the identification and investigation of effective and safe antidiabetic drug from the pharmaceutical compounds which are naturally present as secondary metabolites in plants.

Thus objective of the present study is to investigate the phytochemical and find the *in vitro* antidiabetic activity of various extracts of two plants of the family *Amaranthaceae*, viz., *Digera muricata,* and *Amaranthy cruentus.*

Materials and Methods

Collection of plant material

Fresh leaves of *Digera muricata* and *Amaranthy cruentus* were collected from a location in Coimbatore, Tamil nadu. The disease free and fresh leaves of plant were selected. About 20 grams of fresh and healthy leaves were taken, and washed with tap and distilled water for three times. Surface sterilization was done with 0.1% mercuric chloride for 2 minutes. Again the plant materials were washed thoroughly with distilled water for three times. The leaves were air-dried and pulverized. The plant material was then hermetically sealed in a plastic bag and stored at room temperature until the time of the extraction.

Preparation of extract

These coarse powders of each sample (10 g) were subjected to successive extraction in 100 ml of aqueous, methanol, ethanol, chloroform and acetone. The content was kept in continuous shaker for overnight at room temperature. The respective extracts were filtered twice using Whatman quality filter paper No. 1. The collected extracts were stored at 4 °C and subjected to the qualitative phytochemical analysis and *in vitro* antidiabetic studies.

Qualitative Phytochemical Analysis

Preliminary phytochemical analysis for the two different plant samples were carried out for the five different extract as per standard methods described by Brain and Turner (1975) [3], Evans (1996) [5] and *et al.* (2014).

Alkaloids

About 2 ml of extract was measured in a test tube to which picric acid solution was added. An orange coloration indicated the presence of alkaloids.

Flavonoids

About 4 ml of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated hydrochloric acid was added and red color was observed for flavonoids and orange color for flavones.

Glycosides

About 25 ml of dilute sulphuric acid was added to 5 ml extract in a test tube and boiled for 15 minutes, cooled and neutralized with 10% NaOH, then 5ml of Fehling solution added. Glycosides are indicated by a brick red precipitate.

Proteins

To 2 ml of extract equal volume of 40% NaoH solution and two drops of one percent copper sulphate solution was added. The appearance of violet colour indicates that the presence of protein.

Amino acids

About 2 ml of extract was taken and two drops of freshly prepared 0.2% Ninhydrin reagent was added and heated. The appearance of pink or purple colour indicates that the presence of proteins, peptides or amino acids.

Fats and oil

The test solution was applied on filter paper. It develops a transparent appearance on the filter paper. It indicates that the presence of oils and fats.

Carbohydrates

About 2 ml of extracts was mixed with 5 drops of benedict's solution and kept in water bath for 5 mints. Formations of light green -red-brown in colour presence of carbohydrates.

Saponins

Saponins were detected using the froth test. 1 ml of the sample was measured into a conical flask in which 8 ml of sterile distilled water was added and boiled for 5 minutes. The mixture was filtered and 2.5 ml of the filtrate was added to 10 ml of sterile distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

Phenols

About 2ml extract were treated with few drops of ferric chloride solution. Formation of bluish black colour indicates that the presence of phenol.

Tannins

To a portion of the extract diluted with water, 3-4 drops of 10% ferric chloride solution is added. A blue color is observed for gallic tannins and green color indicates for catecholic tannins.

Steroids

Two ml of acetic anhydride was added to 5 ml of the extracts, each with two ml of H_2SO_4. The colour was changed from violet to blue or green in some samples indicate that the presence of steroids.

Terpenoids

About 5 ml of the extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. An appearance of reddish brown colour in the inner face was indicates that the presence of terpenoids.

Anthocyanin

About 2 ml of the extract was boiled with 10% HCl for few minutes in a water bath. It was allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% NH_3 were added to the mixture and heated. Formation of pink red and turns blue or violet colour indicated that the presence of anthocyanin.

Leucoanthocyanin

The 2 ml of extract mixed with 5 ml of amyl alcohol. A formation of red colour shows presence of leucoanthocyanin.

Coumarins

The 2 ml of extract mixed with 2 ml of sodium hydroxide. A formation of Yellow colour is presence of coumarins.

Emodin

The 2 ml of extract mixed with 2 ml of ammonium hydroxide and add 3ml of benzene. A formation of red color shows presence of emodin.

GC-MS (Gas Chromatography-Mass Spectrometry) analysis

GC-MS analysis was performed to identify some of the potent volatile constituents presents in the extracts. For GC-MS analysis methanol extracts of the powdered *Amaranthus cruentus* and *Celosia argentea* were selected. The analysis was done at The South Indian Textile Research Association (SITRA), Coimbatore, and Tamil Nadu. GC analysis of the extract was performed using a GC-MS (Model; Thermo Trace GC Ultra) equipped with a DB-5MS fused silica capillary column (30 m length X 0.25 mm outside diameter X 0.25 mm internal diameter) and GC interfaced to a Mass Selective Detector (MS-DSQ-II) with XCALIBUR software. For GC-MS detection, an electron ionization system with ionization energy of -70 eV was used. Helium gas was used an a carrier gas at a constant flow rate of 1ml/min and the sample injected was 1 ml; Injector temperature was 260 °C; Ion source temperature was 200 °C. The oven temperature was programmed from 70 °C to 200 °C at the rate of 6 °C/min, held isothermal for 1min and finally raised to 260 °C at 10 °C/min. Interface temperature was kept at 260 °C. Total GC run time was 37.49 min. The relative percentage of the extract constituents was expressed as percentage with peak area normalization.

***In-vitro* antidiabetic activity**

Inhibition assay for α-amylase activity (DNSA):

Five different concentrations of plant extracts were prepared. These were 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml for each of the five extracts in test tubes. A control test tube was prepared which did not contain any plant extract. A total of 500 µl of different plant extract and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/ml) were incubated for 10 minutes at 25 °C. After pre-incubation, 500 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH6.9 with 0.006 M sodium chloride) was added to each tube at 5s intervals. This reaction mixture was then incubated for 10 minutes at 25 °C. About 1 ml of DNSA colour reagent was added to stop the reaction. These test tubes were then incubated in a boiling water bath for 5 minutes and cooled to room temperature. Finally this reaction mixture was again diluted by adding 10 ml distilled water following which absorbance was measured at 540 nm.

Calculation of 50% Inhibitory Concentration (IC_{50})

The concentration of the different plant extracts required to scavenge 50% of the radicals (IC_{50}) was calculated by using the percentage scavenging activities at five different concentrations of the extracts. IC_{50} values were calculated using the online IC_{50} calculator tool of AAT Bioquest (https://www.aatbio.com/tools/ic50-calculator).

Results

Preliminary qualitative phytochemical analysis

The present study revealed that the aqueous, methanol, ethanol, chloroform and acetone extracts of leaf parts of both *Digera muricata* and *Amaranthus cruentus* contained alkaloids, flavonoids, glycosides, amino acids, fatty acids, carbohydrates, saponins, proteins, phenolic compounds, tannins, sterols, terpenoids, anthocyanins, leucoanthocyanins, coumarins, and emodins (Table 1 & 2). However, tannins were detected only in aqueous extracts of *Digera muricata* and emodins were detected only in aqueous extracts of *Amaranthus cruentus*. Both alkaloid and coumarins were completely absent in all the five extracts of *Amaranthus cruentus*. Methanol extracts of both the plants showed the presence of rich variety of secondary metabolites. Whereas aqueous, chloroform, ethanol and acetone extracts showed the less variety of these secondary metabolites. Compared to all other solvent extracts, methanol leaf extracts had higher number of secondary metabolites therefore methanol extract was used for further studies.

Table 1: Qualitative chemical analysis of phytoconstituents of the leaf powder with various extracts of *Digera muricata*

Plant constituents	Aqueous	Ethanol	Methanol	Chloroform	Acetone
Alkaloids	+	+	-	+	+
Flavonoids	-	+	+	+	-
Glycoside	+	+	+	-	+
Amino acids	+	+	+	-	-
Fatty acids	+	+	+	-	+
Carbohydrates	+	+	-	-	+
Saponins	+	+	-	-	-
Proteins	+	-	+	-	-
Phenolic compounds	-	+	+	-	+
Tannins	+	-	-	-	-
Sterols	+	-	+	+	-
Terpenoids	+	+	+	+	-
Anthocyanins	-	-	+	-	+
Leucoanthocyanins	+	-	+	-	-
Coumarins	-	-	+	+	-
Emodins	+	+	+	-	-

Table 2. Qualitative chemical analysis of phytoconstituents of the leaf powder with various extracts of *Amaranthus cruentus*

Plant constituents	Aqueous	Ethanol	Methanol	Chloroform	Acetone
Alkaloids	-	-	-	-	-
Flavonoids	-	+	+	+	-
Glycoside	+	+	+	-	+
Amino acids	+	+	+	-	-
Fatty acids	+	-	+	-	+
Carbohydrates	+	+	+	-	+
Saponins	-	-	-	-	+

Proteins	+	-	+	-	-
Phenolic compounds	+	+	+	+	+
Tannins	+	+	+	+	+
Sterols	+	-	+	+	+
Terpenoids	-	+	+	+	+
Anthocyanins	+	-	-	-	+
Leucoanthocyanins	+	-	+	+	-
Coumarins	-	-	-	-	-
Emodins	+	-	-	-	-

Since the methanol extracts of both the plants showed the presence of rich variety of secondary metabolites they were further used for GC MS analysis. The results pertaining to GC-MS analysis of the methanolic extract of *Digera muricata* and *Amaranthus cruentus* lead to the identification of a number of compounds. These compounds were identified through mass spectrometry attached with GC. The present study helps to predict the formula and structure of 13 different biomolecules from *Digera muricata* and about 12 bioactive components from *Amaranthus cruentus* through GC-MS detection as shown in (Table 3 & 4).

Table 3: Biologically active chemical compounds of methanol extract from *Digera muricata*

R t	Compound name	Molecular formula	Area
3.29	Ethane 1,1-diethoxy	$C_6H_{14}O_2$	35.43
10.67	Phenol, 2-methoxy-4-4-(2-propenyl)	$C_{10}H_{12}O_2$	6.91
11.12	1-Hexadecanol	$C_{167}H_{34}O$	9.79
17.65	Cyclohexanol,1-(2-hexynyl)	$C_{12}H_{22}O$	1.04
18.15	Cyclo pentane tridecanoic acid, methyl ester	$C_{19}H_{26}O_2$	1.63
19.21	Benzyl benzoate	$C_{14}H_{12}O_2$	5.83
19.57	Cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	1.31
20.47	Isopropyl myristate	$C_{17}H_{34}O_2$	4.58
22.23	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	1.18
23.53	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	3.20
25.55	9-octadecanoioc acid,- methyl ester	$C_{19}H_{36}O_2$	8.94
26.04	Heptadecanoic acid, 9-methyl-methyl ester	$C_{19}H_{38}O_2$	6.92
27.32	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	4.54

Table 4: *Biologically active chemical compounds of methanol extract from Amaranthus cruentus*

R t	compound name	molecular formula	area
3.29	Ethane, 1, 1-diethoxy	$C_6H_{14}O_2$	1.53
5.01	Hexadecanol-2-methyl	$C_{17}H_{36}O$	7.09
8.12	Galactopyranone, 6-deoxycyclic	$C_8H_{14}B_2O_5$	6.43
9.73	Butanoic acid, hep[tafluro-, methylester	$C_5H_3F_7O_2$	3.68
10.67	Phenol,2-methyl-4-(2-propenyl)	$C_{10}H_{12}O_2$	4.59
11.12	Hexadecanol	$C_{16}H_{34}O$	7.11
11.12	1-Tetradecanol	$C_{14}H_{30}O$	7.11
20.30	Isopropyl myristate	$C_{17}H_{34}O_2$	8.71
23.51	Hexadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	8.53
25.54	9- Octadecanoic acid-,methyl ester	$C_{19}H_{36}O_2$	7.35
26.03	Heptadecanoic acid, 9-methyl- methy ester	$C_{19}H_{38}O_2$	6.25
31.42	9- Hexadecanoic acid, 9- Octadecenyl	$C_{38}H_{74}O_2$	3.70

Ethane 1, 1-diethoxy, Phenol, 2-methoxy-4-4-(2-propenyl), 1-Hexadecanol, Cyclohexanol,1-(2-hexynyl), Cyclo pentane tridecanoic acid, methyl ester, Benzyl benzoate, Cis-13-Eicosenoic acid, Isopropyl myristate, Hexadecanoic acid, methyl ester, Hexadecanoic acid, ethyl ester, 9-octadecanoioc acid,- methyl ester, Heptadecanoic acid, 9-methyl-methyl ester and Octadecanoic acid, ethyl ester were present in the methanolic extracts of *Digera muricata*. Similarly Ethane, 1, 1-diethoxy, Hexadecanol-2-methyl, Galactopyranone, 6-deoxycyclic, Butanoic acid, hep[tafluro-methylester], Phenol,2-methyl-4-(2-propenyl), Hexadecanol, 1-Tetradecanol, Isopropyl myristate, Hexadecanoic acid, methyl ester, 9- Octadecanoic acid- methyl ester, Heptadecanoic acid, 9-methyl-methy ester and 9- Hexadecanoic acid, 9-Octadecenyl were present in the methanolic extracts of *Amaranthus cruentus.* The GC chromatograms of the two extracts presented in Figure1 & 2 shows the retention time in the column and the detected peaks which correspond to the bioactive compounds present in the extract.

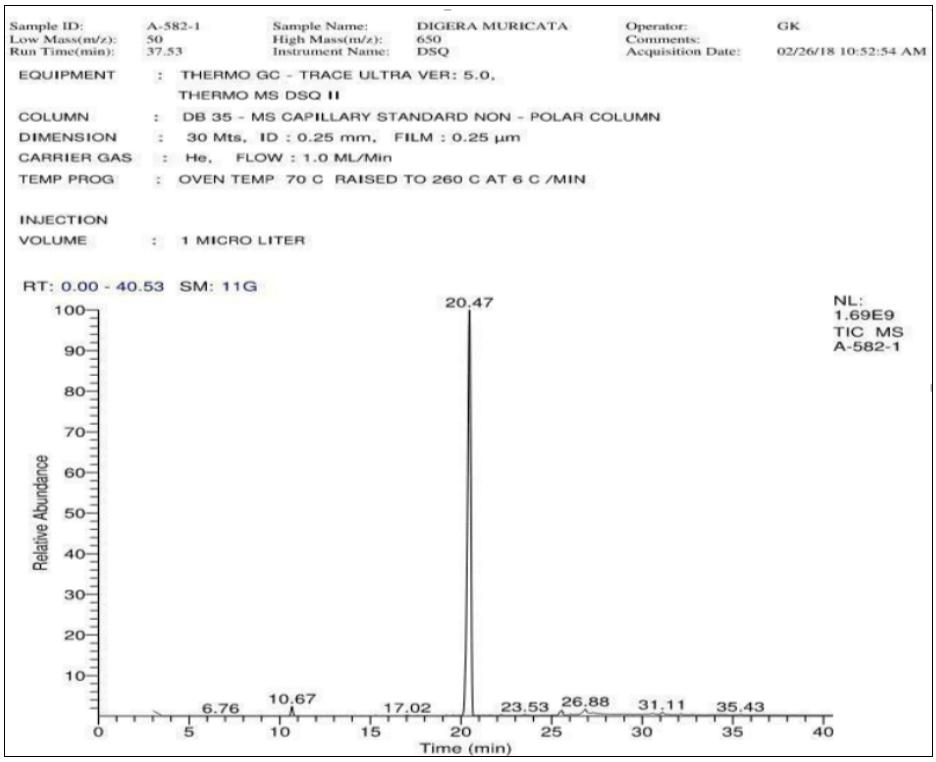


Fig 1: GC-MS chromatogram of methanlic extract of *Digera muricata*

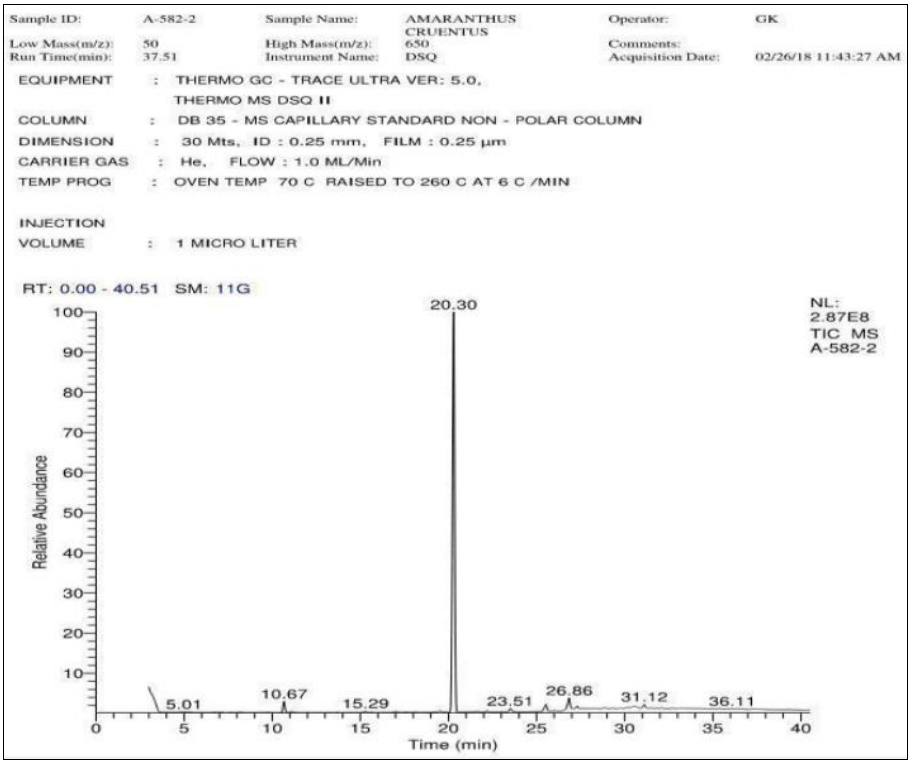


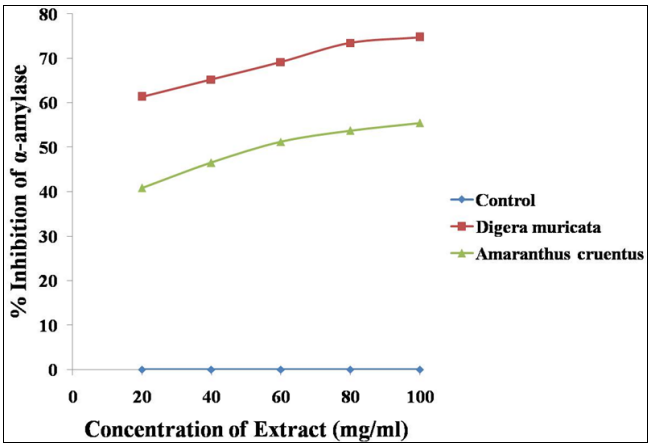
Fig 2: GC-MS chromatogram of methanlic extract of *Amaranthus cruentus*

Inhibition assay for α-amylase activity (DNSA) and IC_{50} Analysis

Table 5 shows the results of α-amylase inhibitory action of the two plant leaf extracts namely *Digera muricata* and *Amaranthus cruentus* at five different concentrations (20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml & 100 mg/ml). Both the plants showed dose-dependent inhibition of α-amylase enzyme with varying effect on glucose utilization. Among the two extracts, *Digera muricata* extracts have shown the highest inhibitory activity when compared with *Amaranthus cruentus.* At all concentrations, *Digera muricata* showed maximum inhibition of the enzyme with the highest value of 74.7% seen at 100 mg/ml concentration of plant extract. *Amaranthus cruentus* showed the highest value of 55.4% seen at 100 mg/ml concentration of plant extract (Graph 1). The IC_{50} analysis is commonly used for quantifying the concentration of the extract which inhibits 50% of enzyme activity. In this study, the methanolic leaves extract of *Digera muricata* showed highest IC_{50} value of about 59.45 compared to *Amaranthus cruentus* that showed IC_{50} value of 46.73. Hence, it was concluded that the methanolic leaves extract of *Digera muricata* is more effective in inhibiting α-amylase activity compared to *Amaranthus cruentus*.

Table 5: Inhibition activity and IC_{50} values of α-amylase activity in *Digera muricata* and *Amaranthus cruentus* at different concentration

Concentration (mg/ml)	Control (%)	*Digera muricata*	*Amaranthus cruentus*
20	0	61.3	40.8
40	0	65.2	46.5
60	0	69.1	51.2
80	0	73.4	53.7
100	0	74.7	55.4
IC_{50} α-amylase		59.456	46.734



Graph 1: Effects of *Digera muricata* and *Amaranthus cruentus* extracts at varying concentrations on α-amylase activity. The figures are in percentage with the control showing 0% inhibition.

Discussion

Several reports have shown that usage of antidiabetic drugs causes severe side effects (Saxena & Vikram, 2004; Chiasson *et al*., 2002; Yeh *et al*., 2003) [12, 4, 14]. Therefore it become necessary to identify and isolate such bioactive compounds from the natural resources like medicinal plants which can be effectively used for treating diabetes with less or no side effect. In our current research we have examined the phytochemical constituents and further effective extracts were used for studying the in vitro antidiabetic activities study with different concentrations and the results were compared. From the results, the aqueous, methanol, ethanol, chloroform and acetone extracts of leaf parts of both *Digera muricata* and *Amaranthus cruentus* contained alkaloids, flavonoids, glycosides, amino acids, fatty acids, carbohydrates, saponins, proteins, phenolic compounds, tannins, sterols, terpenoids, anthocyanins, leucoanthocyanins, coumarins, and emodins. Similarly Benedec *et al*., 2013 [2] also reported that many biological and therapeutic properties are abundantly present in the medicinal plants and thus they can be effectively used for treating various diseases. In this current work we could find that methanol was one of the best solvent as high numbers of compounds were identified when compared with the other four solvents used. This is in agreement with the previous reports of Paulsamy *et al* (2011) [10]. Further the GC-MS spectrum confirmed the presence of various components with different retention times as reported by Lakshmi *et al*., 2014 [7].

At all concentrations, *Digera muricata* showed maximum inhibition of the enzyme with the highest value of 74.7% seen at 100 mg/ml concentration of plant extract. *Amaranthus cruentus* showed the highest value of 55.4% seen at 100mg/ml concentration of plant extract. Our results are in accordance with the previous study wherein, both the selected plants showed positive results for the presence total polyphenol and flavonoid content through phytochemical study (Ramkumar *et al*., 2010; Manikandan *et al*., 2013) [11, 8]. Further, the methanolic leaves extract of *Digera muricata* showed highest IC_{50} value of about 59.45 compared to *Amaranthus cruentus* that showed IC_{50} value of 46.73 for α-amylase enzyme. Therefore the study clearly says that both these plants can be effectively used for developing anti-diabetic drug with no side effects.

Conclusion

The presence of various bio-active compounds detected in *Digera muricata* and *Amaranthus cruentus* by phytochemical and GC-MS analysis validates the presence of phytochemical constituents. Compared with *Amaranthus cruentus*, *Digera muricata* showed to have significant enzyme inhibitory activity. Therefore further isolation and characterization of individual biomolecules through *in vivo* studies may result with an effective anti-diabetic drug with very no side effect.

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