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Anti-diabetic Effect of Ethanolic Extracts of Cissus Quadrangularis linn Fruits and Michelia Champaea Leaves in Alloxan Induced Hyperglycaemic Rats and in 3T3L1 Cell Lines

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ABSTRACT

Background: Diabetes is a metabolic disorder that results in increased blood sugar. This study came up with a search for alternative medicines with no or fewer side effects for antidiabetic therapy.

Materials and methods: The plants are extracted and screened for phytochemical study of crude extracts. Anti-diabetic activity in alloxan induced hyperglycaemic rats, in single dose study, multiple dose treatment was investigated. *In vitro* cellular assay using 3T3L1 cell line was performed to check the cell viability with increasing plant extract treatment.

Results: Phytochemical investigation reveals the presence of alkaloids, flavonoids, saponins, tannins, steroids, triterpinoids, carbohydrates, and glycosides in both plant extractions. In acute toxicity studies, no mortality was observed with either of the extracts even at a dosage of the level of 5000 mg per kg of body weight. The ethanolic extracts showed a noteworthy decline in blood glucose level in alloxan induced rats in both single and multiple dosage methods. Significant changes were observed in the serum glucose and body weight from day 0 to day 14. The cell viability of the extracts was also comparable with the standard.

Conclusion: Our results report that CQ fruits and MC leaves have potent antidiabetic action and on further studies, can be a credible resource in antidiabetic therapy.

Key Words: *Cissus Quadrangularis Linn Fruits, Michelia Champaea Leaves*, Anti-diabetic activity, 3T3L1 cell line, Cyototoxicity, Wistar rats.

INTRODUCTION

Diabetes mellitus (DM) is a syndrome characterized by chronic hyperglyceamia and disturbances of carbohydrate, fat, and protein metabolism associated with absolute or relative deficiencies in insulin secretion and/or insulin action[1]. It is a metabolic disorder comprising of micro and macro vascular complications that result from insignificant morbidity and mortality. It is a major cause of death worldwide [2]. There are an estimated 143 million people worldwide diagnosed with DM and this number will probably double by the year 2030 [3].

Despite significant advancement in the treatment of DM using oral hypoglycemic agents, exploration for newer drugs continue due to several limitations of synthetic drugs. In recent times, there has been a renewed interest in plant remedies [4-5].

The medicinal properties of CQ were known since the distant past. Cissus was also used in ayurvedic medicines for treating injured bones, ligaments, and tendons. In Siddha medicine CQ finds its application as an analgesic, and in treating broken bones. The *Magnoliaceae* belong to the fossil plant family dated back to 95 million years. These are characterized by large, cup-shaped flowers with no distinct petals. Some species, including the champak *(Michelia champaca)* and *Michelia doltsopa* are grown for their flowers, both on the tree and as cut flowers. Champak flowers are also used to produce essential oil for perfume. A few species have been introduced to gardens or as street trees outside of the Indomalaya region, including *Michelia figo*, *M. doltsopa*, and *M. champaca*[6-8].

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The current paper deals with screening of *Cissus quadrangularis linn* fruits and *Michelia champaea leaf* extracts for anti-diabetic activity in alloxan induced hyperglycaemic rats.

MATERIALS AND METHODS

Materials

Experimental Animals

Adult Wistar albino rats (150-200 g), housed in the institutional animal house and used for the study. Animals caged in polypropylene cages in a controlled environmental condition (22± 3^0C, 55 ± 5% humidity, and a 12 h light/ dark cycle). The animals were provided with a regular rodent diet and water *ad libitum*. The animals were allowed to adapt to these conditions for a week.

Methods

Plant collection and authentication

The CQ fruits and MC leaves were obtained from the local places of Tirupati, AP. *The CQ* fruit was authenticated by Dr. K. Madhava Chetty, M.Sc., M.Ed., M.Phil., Ph.D., PG DPD., Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh.

Extraction by maceration

Fresh leaves of *MC and fruits of CQ* were washed with water to get rid of contaminants like dirt and other impurities and were shade-dried. These dried leaves and fruits were ground and sieved to get a uniform, coarse powder. Powdered plant material was weighed (1Kg) and immersed in 95% ethanol and kept for maceration for 7 days with occasional stirring. On the 8^{th} day, the solvent was filtered by pressing with a muslin cloth and was evaporated in a rotary evaporator at 40^oC. The resultant extract was put in a desiccator to remove any ethanol left in it. The dried ethanolic extract of *Michelia Champaea* (EEMC) and ethanolic extract of *Cissus Quadrangularis* (EECQ) were packed in an air-tight bottle and put in a dry place for further studies.

Qualitative evaluation of phytoconstituents

The EECQ were screened for the presence of various phytoconstituents like carbohydrates, flavonoids, polyphenolic compounds, saponins, tannins, triterpenoids, etc.

Cell culture

3T3L1 cell line was procured from the National Centre for Cell Science, Pune. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (high glucose) with 10% FBS (Invitrogen, Canada), 10,000 U Penicillin G, 10,000 µg/mL streptomycin sulfate (Invitrogen), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Cultures were maintained at 37°C in 5% CO2 in a humidified incubator.

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay to determine the toxicity of a compound on the cells based on the conversion of MTT to formazan crystals by the lactate dehydrogenase present in live cells [17,18]. The 3T3L1 cells were seeded at an initial density of 20 × 104 cells per well/200 µL in 96 "well" plate and cultured overnight. The cells were then treated with desired concentrations of plant extract (25–400 µg/mL) for 24 h in the same culture conditions. Post-treatment, the medium was aspirated, 0.5 mg/mL of MTT reagent was added to cells and incubated at 37°C for 2 h. MTT reagent was then removed, and formazan crystals were dissolved with 20 µL of dimethyl sulfoxide (DMSO). Absorbance at 570 nm was measured by a microplate reader. Percentage viability was determined using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD of the test at 570 nm}}{\text{Mean OD of Untreated cells at 570 nm}} \times 100$$

Evaluation of anti diabetic activity[9, 10]

For the study of anti-diabetic activity of various extracts of EEMC and EECQ, the study was divided into two phases

(I) Activity in normoglycaemic animals
(II) Activity in alloxan induced hyperglycaemic rats
  (a) Single-dose study
  (b) Multiple-dose study (14 days treatment)

Activity in normoglycaemic animals

Method of oral administration of extracts

An 18-gauge needle was suitably covered with flexible polythene tubing, where the edge was made blunt. The needle was fixed to a 1ml tuberculin syringe. The rat was held firmly in the left hand. The tubing was moistened with glycerin and inserted right into the oesophagus and gently pressing plunger for drug administration, and this was followed by 0.2ml of distilled water to ensure administration of the correct dose of the drug.

Experimental design

Twenty-four animals were categorized into four equal groups. Animals (Wister rats) were fasted for 18 h but were allowed free access to water before and throughout the experiment. The study was approved by the institutional animal ethical committee with No: 1447/PO/Re/S/11/CPCSEA/15/A.

Group-I: Administered with vehicle (distilled water) & served as Normal control.

Group-II: Administered with standard drug Glipizide (5mg/kg).

Group III : Administered EECQ (100mg/kg).

Group IV: Administered EECQ (200mg/kg).

Group V: Administered EEMC (200mg/kg).

Group VI: Administered EEMC (400mg/kg).

Blood samples were withdrawn from the **retro-orbital venous plexus** with capillary tubes under ether anesthesia and with Sodium citrate as an anticoagulant. Serum was separated by centrifugation. The glucose level in blood was measured after 0, 1, 2, 4, 8, 12& 24 h of administration of the single dose of test samples.

Estimation of serum glucose by GOD/POD method[11]

This method utilizes two enzymes Glucose Oxidase (GOD) and Peroxidase (POD) along with chromogen-4-amino antipyrine, phenol and is intended for in vitro quantitative determination of glucose in serum, plasma, and cerebrospinal fluid. There was no interference due to the creatinine, fructose, galactose, reduced glutathione, ascorbic acid, and xylose. Hemoglobin or bilirubin up to 10mg does not affect the test.

Single Dose Study [12]

Induction of diabetes

The animals were allowed to fast for 24 h and rendered diabetic by injecting a single dose of alloxan at 150mg/kg body weight administered as a 5% w/v in distilled water by i.p. route. It produces diabetes by selected necrosis of b - cells of islets of Langerhans of the pancreas.

After 48 h of injecting alloxan, diabetes was confirmed by testing blood sugar with *Erba CHEM 5 Plus* Auto analyzer.

The animals with a sugar level of more than 250mg/dl were selected. Animals were maintained for four days in diabetic conditions for good establishment of diabetes.

Standard: Glipizide at the dose of (5mg/kg) was used as a standard drug.

Experimental Design: Animals were categorized into 5 different groups of six animals each. The animals (Wister rats) were fasted for 18 h but were allowed free access to water before and throughout the experiment.

Blood samples were collected from the retro-orbital venous plexus with capillary tubes under ether anesthesia and with sodium citrate as an anticoagulant. Serum was separated by centrifugation.

Multiple-dose treatment (14 days treatment)

The animals used for this study are the same animals used for the single-dose study, had free access to feed and water during this period.

The chronic study involved repeated administration of extracts of EEMC, EECQ, and Glipizide for 14 days(once a day) to the groups used for single-dose study at a prefixed time and the glucose levels in blood estimated in samples withdrawn after 2 h on day 0,7th and 14th day.

Statistical analysis

The result analysis was carried out by the one-way ANOVA method followed by Dunnett's multiple comparison tests.

RESULTS

Preliminary phytochemical screening

Results of phytochemical screening were elucidated in **Table-1.**

The preliminary phytochemical screening indicated the presence of various phytoconstituents like flavonoids, phenolic compounds, triterpenoids, tannins, saponins, amino acids, proteins, and carbohydrates in *EECQ*. The preliminary phytochemical screening showed the presence of various phytoconstituents like flavonoids, phenolic compounds, triterpenoids, tannins, saponins, amino acids, proteins, and carbohydrates in *EEMC*.

Cytotoxicity assay on 3T3 L1 cell line

3T3-L1 cells were treated with different concentrations (25 µg–400 µg/mL) of *EECQ and EEMC* were assayed for their cytotoxic effect. The extract displayed no cytotoxic effect on cells. The concentrations of the extract used and the respective percent cell viability were tabulated and plotted [Table 2 and Figures 1].

Body weight

The changes in body weight of the different groups of animals during the period of study was given in **Table 3** and represented in **Figure 2** which shows an increase in the mean body weight (± SEM) of normal rats from 230.33 ± 1.47g on day 0 to 240.00 ± 1.06g on day 7, 249.2 ± 0.94g on day 14. This shows that the group of normal rats gained body weight during the treatment period of 14 days.

Effect on normoglycaemic rats

The fasting serum glucose of the different groups of animals during the single-dose treatment period of study is given in **Table 4** and presented in **figure 3.** which shows that the mean (±SEM) fasting serum glucose values of the normal group of rats was 95.16 ± 1.81, 95.16 ± 1.078, 95.83 ± 1.49, 96 ± 1.00, 97.33 ± 1.60, 96 ± 0.85 and 95.83±0.60 mg/dl, on 0, 1, 2, 4, 8, 12 and 24 h respectively.

Effect on alloxan induced hyperglycaemic rats

The fasting serum glucose of the different groups of animals during the single-dose treatment period of study is given in **Table 5**. and presented in **figure 4**, which shows that the

mean (±SEM) fasting serum glucose values of the normal group of rats was 95.76 ± 3.248, 96.2 ± 3.21, 96.86 ± 3.47, 97.3 ± 3.26, 97.96 ± 3.29, 98.65 ± 3.15 and 97.68±3.44 mg/dl, on 0, 1, 2, 4, 8, 12 and 24 h respectively.

Multiple-dose study

The fasting serum glucose of the different groups of animals during the chronic study is given in **Table 6** and presented in **figure 5**. which shows that the mean (±SEM) fasting serum glucose values of the normal group of rats was 94.63±3.54,96.27 ±3.60, 97.29±3.09 mg/dl on day 0, day 7, and day 14 respectively. The above values show that the fasting serum glucose in the normal group of rats was maintained within the normal range throughout the study.

DISCUSSION

In cytotoxicity assay on 3T3 L1 cell line, the lowest concentration of EECQ and EEMC (25 µg/mL) showed 98.6% and 99.7% viability respectively, and the highest concentration (400 µg/mL) showed 89.45% and 88.9% of viability respectively after 24 h of exposure. These results indicated that EECQ and EEMC are not toxic to mammalian cells even at higher concentrations and could be used to analyze other parameters of antidiabetic studies. Metformin (100 µM) treatment – positive control – also had a percent viability of 97.4% post 24-h exposure[13,14]. During the period of treatment, the diabetic group of rats has shown a change in body weight from a mean (± SEM) value of 190.5 ± 1.2g on day 0, 160. ± 1.28g. On day 7 and which decreased further to 132.8 ±1.07g on day 14. The glipizide (5 mg/kg) treated group body weight was found to have been increased. The body weight gain in this group of rats from day 0 through day 7 to day 14 was relatively less when compared with the normal group[15-17]. The EECQ (100 mg/ kg) treated group of diabetic rats was found to have a mean body weight (± SEM) of 174.3±2.30 g on day 0, 178.00±1.4 g on day 7, 202.3±0.9 g on day 14 . The EECQ (200 mg/kg) treated group of diabetic rats shows mean (± SEM) body weight of 164 ±1.25 g on day 0, 172.80 ± 1.9 g on day 7, 195.80 ± 1.13 g. on day 14. The EEMC (200 mg/kg) treated group of diabetic rats shows mean (± SEM) body weight of 161±1.12g on day 0, 169±2.21g on day 7, 194±2.11g on day 14. The EEMC (400 mg/kg) treated group of diabetic rats shows mean (± SEM) body weight of 158±1.23g on day 0, 165±1.43g on day 7, 192±1.44, on day 14.

Effect on normoglycaemic rats was studied by measuring glipizide (5 mg/kg) treated normal rats show a mean (±SEM) fasting serum glucose of 96.83 ± 0.60, 94 ± 1.29, 86 ± 1.29, 79.16 ± 0.94, 74 ± 1.03, 69.66 ± 0.33 and 92.16 ± 0.83mg/dl on 0, 1, 2, 4, 8, 12 and 24h respectively. The EECQ and EEMC treated normal rats showed mean fasting serum glucose of reduced levels respectively with doses. These changes in fasting serum glucose values illustrate that the normoglyceaemic rats treated with EECQ and EEMC show a progressive and significant reduction. [18]

Effect on alloxan induced hyperglycaemic rats was studied by measuring mean fasting serum glucose (±SEM) in the diabetic control group of rats was found to be 262.16 ± 07.96, 266.5 ± 7.39, 275.5 ± 7.20, 285 ± 7.42, 296.16 ± 6.84, 316.5 ± 4.61 and 326.16 ± 4.76mg/dl on 0, 1, 2, 4, 8, 12 and 24 h respectively, which was found to be significantly ($p \leq 0.01$) higher when compared with the normal rats. [19]

In the multiple dose study the mean fasting serum glucose (±SEM) in the diabetic control group of rats was found to be 269.64±2.89, 337.73±9.899 and 386.5±17.92 mg/dl on 0^{th}, 7^{th} and 14^{th} day respectively, which was found to be significantly ($p \leq 0.01$) higher when compared with the normal rats. These elevated fasting serum glucose levels were found to have been maintained throughout the 14 days of the treatment period indicating that the rats are rendered diabetic. The glipizide (5 mg/kg) treated diabetic rats show a mean (±SEM) fasting serum glucose was reduced from day 0 to day7 and then to day 14, a similar reduction was also observed with multiple doses treated groups.[20]

CONCLUSION

Phytochemical evaluation of EECQ and EEMC showed the presence of carbohydrates, flavonoids, tannins, terpenoids, saponins, proteins, amino acids, and phenolic compounds. According to the literature, EECQ and EEMC were found to be safe in the dose used and there was no mortality up to 5000 mg/kg dose.

The results indicate that the EECQ fruits *and EEMC leaves* have good anti-diabetic activity. The ethanolic extracts of CQ fruits and MC leaves displayed noteworthy anti-hyperglycaemic activity in alloxan-induced hyperglycaemic rats without any major variation in body weight; they also enhanced the condition of DM that was indicated by body weight, serum creatinine, serum urea, and serum alkaline phosphatase. The study also displayed damage of the pancreas in alloxan-treated diabetic control rats and leads to regeneration of cells by glipizide and extract treatment group.

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Conflict of Interest

No conflict of interest

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Authors' Contribution

Jasti Deepthi developed the theoretical formalism, performed the analytic calculations and numerical simulations. Both Jasti Deepthi and D V R N Bhikshapathi contributed to the final version of the manuscript.

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Table 1: Results of phytochemical screening of EECQ

S. No	Name of the phytochemical	EECQ	EEMC
1.	Carbohydrates	+	+
2.	Amino acids	+	+
3.	Proteins	+	+
	Alkaloids	+	+
	Cardiac glycosides	+	+
6.	Triterpenoids	+	+
7.	Saponins	+	+
8.	Flavonoids	+	+
	Phenolic compounds	+	+
10.	Tannins	+	+
11.	Steroids	-	-
12.	Gums	-	-

Where, + means positive and - means negative.

Table 2: Percentage viability on various concentrations of EECQ and EEMC

EECQ		EEMC	
Treatment groups	Percentage of viability	Treatment groups	Percentage of viability
Control	100	Control	100
Metformin 100 µg/ml	97.4	Metformin 100 µg/ml	97.4
25 µg/ml	98.6	25 µg/ml	99.7
50 µg/ml	98.0	50 µg/ml	97.1
100 µg/ml	95.6	100 µg/ml	96.5
200 µg/ml	93.6	200 µg/ml	94.3
400 µg/ml	90.45	400 µg/ml	91. 9

Table 3: Effect of EECQ and EEMC on body weight of rats

Groups	Dose Administered	Body Weight (Mean ± S.E.M) in 'gm'		
		0 Day	7th Day	14th Day
Group-I	Distilled water	230.33±1.47	240.00±1.06	249.2±0.94
Group-II	Allaxon,150mg/kg	190.5±1.2[a]	160.6±1.28[a]	132.8 ± 1.07[a]
Group-III	Glipizide 5mg/kg	189.73±2.4*	204.6±1.08*	212.5±1.6*
Group-IV	EECQ 100mg/kg	174.3±2.3*	178.00±1.4*	202.3±0.9*
Group-V	EECQ 200 mg/kg	164±1.25*	172.8±1.9*	195.8±1.13*
Group-VI	EEMC 200mg/kg	161±1.12*	169±2.21*	194±2.11*
Group-VII	EEMC 400mg/kg	158±1.23*	165±1.43*	192±1.44*

Values are expressed as mean ± SEM; n=6

[a] p≤0.01 Diabetes control Vs G-I

* p≤0.05, Glipizide/MEBR/MECA/MEMP/PHME Vs Diabetic Control.

Table 4: Effect of EECQ and EEMC on serum glucose levels in normoglycaemic rats

Groups	Dose (mg/kg)	Serum glucose levels in mg/dl (Mean ± S.E.M)						
		0 h	1h	2h	4h	8h	12h	24h
Group-I	Distilled water	95.16±1.81	95.16±1.07	95.83±1.49	96.00±1.00	97.33±1.60	96.00±0.85	95.83±0.6
Group-II	Glipizide 5mg/kg	96.83±0.60	94.00±1.29**	86±1.29**	79.16±0.94**	74 ± 1.03**	69.66±1.25**	92.16±0.83**
Group-III	EECQ 100mg/kg	96.16±1.22	96.00±1.12*	92±0.73*	85.0±1.03*	79.16± 2.27*	76.00 ± 0.57	92.25± 0.79*
Group-IV	EECQ 200 mg/kg	97.16±0.7	96.00±0.57	95.16±1.72*	89.16±1.35*	75.83±1.53*	83.33±1.20*	90.33±1.17*
Group-V	EEMC 200mg/kg	96.12±1.12	95.31±1.11	94.22±1.34	88.16±1.24	79.24±1.15**	68.15±1.16	91.11±1.21
Group-VI	EEMC 400mg/kg	96.32±1.32	94.31±1.15	92.42±1.17	87.15±1.11	78.11±1.32**	67.16±1.23	91.21±1.33

Values are expressed as mean ± SEM; n=6. **P<0.05, **P<0.01,* compared to G-I

Table 5: Effect of EECQ and EEMC on serum glucose levels in alloxan induced diabetes rats (Single dose study)

Group	Dose (mg/kg)	Serum glucose levels in mg/dl (Mean ± S.E.M)						
		0 hr	1 h	2h	4h	8h	12h	24h
I	Water	95.76 ± 3.24	96.2 ±3.21	96.86 ±3.47	97.3 ±3.26	97.96 ±3.29	98.65 ±3.15	97.68 ±3.44
II	alloxan-150mg/kg	262.16±7.96 b	266.5±7.39 b	275.5±7.2 b	285±7.42 b	296.16±6.84 b	316.5±4.61 b	326.16±4.76 b
III	Glipizide 5 mg/kg	253±3.36	237.83±5.36** a	168.16±2.79** a	83±4.09** a	87.5±3.48** a	95.00±2.06** a	98.35±1.75** a
IV	EECQ 100mg/kg	265.83±5.19	238.83±3.67** a	195.33±2.29** a	138.33±1.92** a	98.16±1.85** a	123.91±2.25** a	140.65±1.80** a
V	EECQ 200 mg/kg	268.36±6.75	249.8±5.14	207.25±3.0** a	156.58±6.56** a	112.26±3.76* a	131.05±3.47** a	177.51±7.35** a
VI	EEMC 200mg/kg	258.11±2.33	242.21±1.32	174.23±2.21	88.22±2.32	95.11±2.16** a	101.21±2.22	106.13±2.32** a
VII	EEMC 400mg/kg	255.11±1.45	239.21±1.61	171.21±1.23	86.31±1.14	89.11±1.32** a	97.23±2.34	101.11±1.33** a

Values are expressed as mean±SEM; n=6 *$P<0.05$, **$P<0.01$,
'**a**' indicates comparison of G-III, IV and G-V with Diabetic control **$P<0.01$ '**b**' indicates comparison of G-II with G-I

Table 6: Effect of EECQ and EEMC on serum glucose levels of rats after multiple dose treatment

Groups	Dose (mg/kg)	Serum glucose levels in mg/dl (Mean ± S.E.M)		
		0 Day	7 Day	14 Day
I	Distilled water	94.63±3.54	96.27±3.60	97.29±3.09
II	Alloxan 150mg/kg	269.64±2.89a	337.73±9.89a	386.51±17.92a
III	Glipizide 5mg/kg	251.83±3.07*	158.30±0.55**	106.6±0.98**
IV	EECQ 100mg/kg	271.26±3.68	185.11±6.63**	123.19±2.25**
V	EECQ 200 mg/kg	274.11±2.48*	189.48±5.56**	130.31±1.79**
VI	EEMC 200mg/kg	254.45±2.34	163.11±2.44**	109.32±1.34**
VII	EEMC 400mg/kg	253.11±3.41	161.43±3.55**	107.45±4.52**

Values are expressed as mean ± SEM; n=6 [a] p≤0.05 Diabetes control Vs Normal control.**p≤0.01, Vs Diabetic Control

Metformin 100 µg/ml	EECQ 400 µg/ml	EEMC 400 µg/ml

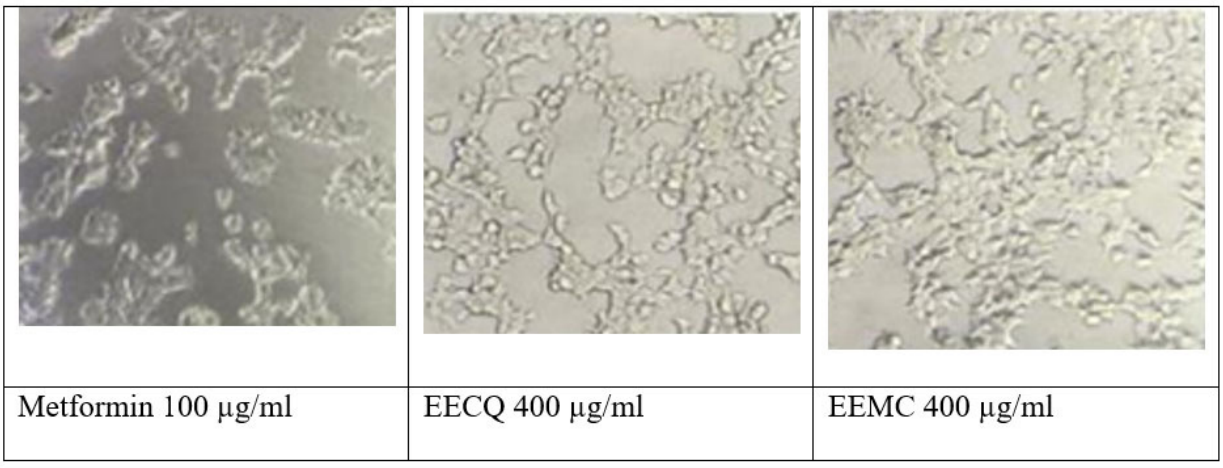

Figure 1: Photos representing various concentrations of EECQ and EEMC on 3T3 L1 cell line.

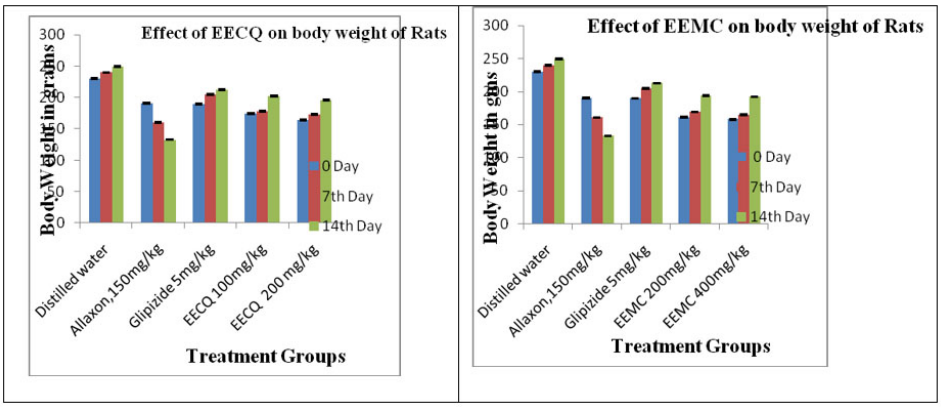

Figure 2: Effect of EECQ and EEMC on body weight in rats.

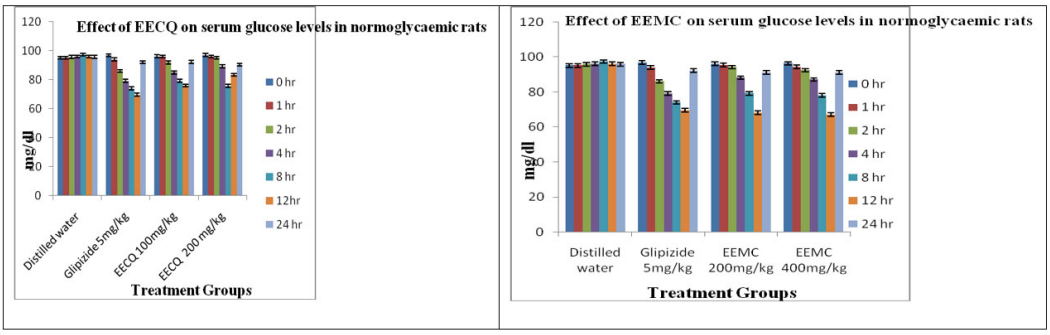

Figure 3: Effect of EECQ and EEMC on serum glucose levels in normoglycaemic rats

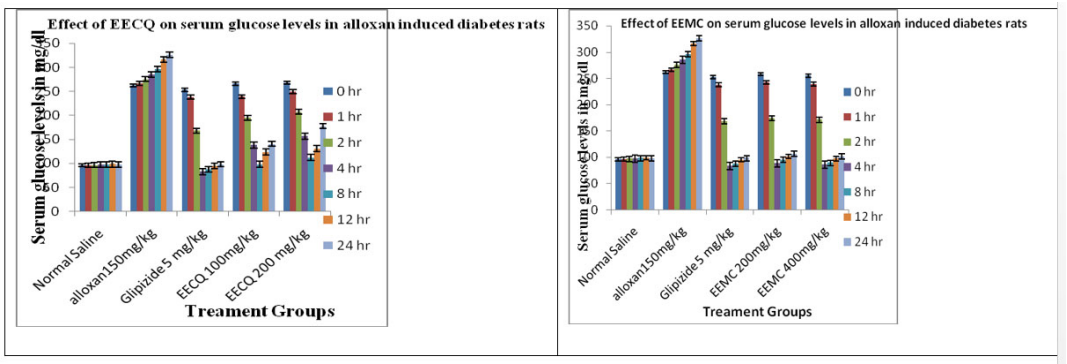

Figure 4: Effect of EECQ and EEMC on serum glucose levels in alloxan induced diabetes rats (Single dose study)

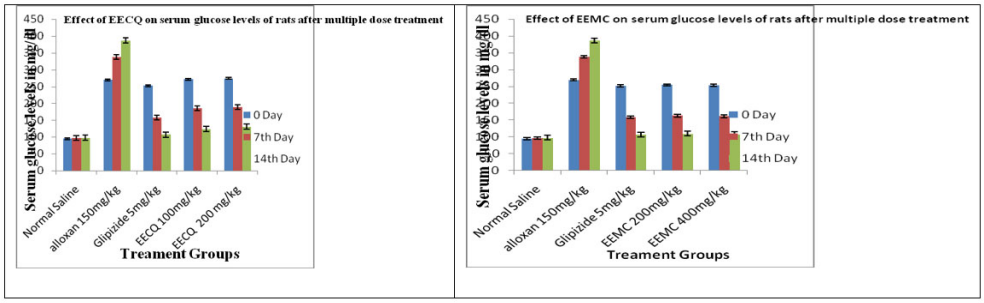

Figure 5: Effect of EECQ and EEMC on serum glucose levels of rats after multiple dose treatment.