Research Paper

Phytochemical Analysis and Hypoglycemic Potential of *Exacum bicolor* Roxb. in Diet Induced Obese Mice

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***Exacum bicolor* Roxb., is popularly documented in folklore medicine for treatment of diabetes mellitus besides other ailments such as fever, inflammation, diseases related to skin and eyes. In this present investigation, diet induced obese mice were treated with aqueous extracts of *in vitro* propagated *Exacum bicolor* plantlets at three different concentrations of 1, 10, and 100 mg/ kg body-weight of mice/day respectively. Diet induced obese mice treated with Glibenclamide 2 mg/kg body-weight of mice/ day were used as a standard. The results displayed reduced blood glucose, triglycerides, cholesterol and free fatty acids in a dose dependent manner in the diet induced obese mice treated with aqueous extracts of *in vitro* propagated *Exacum bicolor*. The results were supported by the histopathological studies of the extracts showing their ability to restore the acini architecture and beta cell number in pancreatic tissues. Further, the study was extended comparing the pharmacological properties such as alpha amylase binding assay between the *in vitro* and native plant extracts of the *Exacum bicolor*. The *in vivo* and *in vitro* studies of the current research validated and confirmed enhanced antidiabetic properties of the aqueous extracts of *in vitro* propagated plantlets over the methanolic extracts from native *Exacum bicolor* plants, corroborating the *in vitro* propagation and their applications in drug development.**

Key words: Alpha amylase, anti-diabetic, diet induced obese mice, *Exacum bicolor*, *in vitro* propagation

Diabetes is a metabolic disorder resulting from altered glycemic conditions acquired either genetically or through induced factors such as obesity, physical inactivity, high glycemic diet, smoking, alcohol consumption and lifestyle changes etc.,[1]. According to the World Health Organization (WHO), November 2021, about 422 million people globally have diabetes, the majority living in lower and middle-income countries. 1.5 million deaths are directly attributed to diabetes each year. It is reported that 95 % of diabetics have Type 2 Diabetes (T2D)/ diabetes mellitus[2]. Hyperglycemic conditions of T2D are highly prevalent and are mainly caused by the deficiency in the pancreatic cells to secrete insulin in adequate quantity or due to insulin resistance[3]. If untreated, this could lead to oxidative stress, inflammation and vascular complications such as nephropathy, neuropathy, cardiac diseases, retinal damage and reduced wound healing abilities. Also, a strong relationship is reported between obesity and diabetes wherein obesity leads to insulin resistance and onset of diabetes making them atopic and vulnerable to other medical complications[4-6].

Ethnobotanical literature reports the use of numerous medicinal plants and their crude extracts in the traditional treatment of diabetes[7,3,8]. *Exacum bicolor* (*E. bicolor*) Roxb., a member of the Gentianaceae family is an endemic of Western Ghats, used as a folklore medicine in treatment of diabetes, fever, haemorrhage, colitis, inflammation and skin burns[9-11]. Earlier reports on the phytochemical investigation of the plant revealed the presence of secondary metabolites viz., glycosides, alkaloids, tannins, flavonoids, saponins, triterpenes and steroids[12-15]. Exhaustive usage of this plant in traditional medicine, increased urbanization in

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Accepted 27 February 2025
Revised 29 November 2024
Received 09 May 2024
Indian J Pharm Sci 2024;87(1):44-50

addition to the high degree of seed dormancy has led to the development of *in vitro* propagation techniques. In the present study, the antidiabetic property of the aqueous extracts of *in vitro* generated *E. bicolor* plantlets was tested on Diet Induced Obese (DIO) mice validating on par with the medicinal properties of the native plant. These results were compared to correlate with histopathological studies. The effective dosage identified was further used in determining the antioxidant and alpha amylase binding assays of the *in vitro* and native plant extracts.

MATERIALS AND METHODS

E. bicolor plant material was collected from Kaagineeri gudda in Sakaleshpura (Western Ghats-12°, 7968.70 N, 75°, 6548.10 E), India during August to September (flower/fruit development stage). The plant specimen RRCBI-4009 was authenticated and certified by National Ayurveda Dietetics Research Institute (NADRI) in Bengaluru. All the reagents used are of analytical grade and procured from Himedia, SD Fine Chemicals and SRL biochemicals. Glibenclamide used as a standard anti-diabetic drug was obtained from Sigma Aldrich (PHR1287-1G).

In vitro propagation:

The *E. bicolor* plantlets were developed as described by Sharad *et al.*[16]. Well-developed plantlets were harvested and used in this study.

Crude extract preparations:

The 3 mo-old *in vitro* cultured plantlets and the aerial parts of native *E. bicolor* plant were collected and shade dried for a week and manually powdered. Aqueous and methanolic extracts were prepared by dissolving 100 mg/ml of powdered plant material with respective solvents and placed in a shaker incubator for 72 h. Aqueous and methanolic crude extracts were soxhlet extracted, concentrated under reduced pressure and stored for comparative pharmacological studies[13].

Alpha amylase binding assay:

The aqueous and methanolic extracts of *in vitro* propagated and native plants were tested for their binding affinity towards alpha amylase by fluorescence spectroscopy[17]. 3 U/ml of alpha amylase solution was prepared freshly and used in this analysis. Acarbose at 1 mg/ml was used as standard. 0.5 ml of standard and the crude extracts were separately mixed with 0.5 ml of 3 U/ml of freshly prepared alpha amylase solution. This mixture was incubated for 5 min in the dark and later, the emission spectra at 282 nm were recorded from the Perkin Elmer ES55 fluorescence spectrometer.

Anti-diabetic activity of crude extracts of *in vitro* and native plants on DIO mice:

DIO mice C57BL/6J were considered for testing the anti-diabetic property of the aqueous extracts from *E. bicolor* cultured plantlets. The permission to use C57BL/6J mice as animal models was obtained from the Institutional Animal Ethics Committee (IAEC). A total of thirty six male C57BL/6J mice aged around 7 w to 9 w (20 g-25 g) were procured from Charles River United States of America (USA). All these mice were initially acclimatized for a week at temperature; 20.2 to 22.8 humidity; 46-69 %, air changes; 12-16 changes per hour, light cycle; 12 h light and 12 h dark cycle following the guidelines laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Grouping and inducing obesity:

After a week of acclimatization, the mice were divided into 6 groups with 6 mice in each group based on body weight stratification and randomization method. To induce the obesity through diet, the mice in the five groups (30) were fed with food rich in carbohydrates and fats (43.77 %, carbohydrates: 22.23 %, proteins: 20.28 %, crude fibres: 6.52 %, ashes: 6.55 %, moisture: 0.65 % and the reverse osmosis purified water was provided *ad libitum*).

Study design:

Six groups of C57BL/6J mice each having six mice were taken for the study. One group (G0) was labelled as control and the remaining five groups were induced with obesity as mentioned above leading to onset of hyperglycaemic conditions. The blood and lipid parameters were monitored for all the mice initially (0^{th} d) and after inducing obesity through diet (30^{th} d). The obese mice were treated with glibenclamide and aqueous extracts of *in vitro* propagated *E. bicolor* plantlets at three different concentrations (1 mg/kg, 10 mg/kg, and 100 mg/kg body weight of mice) for 4 w as follows: Group 1 (G1) was considered as DIO control with the DIO mice not receiving any treatment for their hyperglycemic conditions; group 2 (G2) the DIO mice treated with glibenclamide at 2 mg/kg of body weight (bw)/day; group 3-5 (G3-G5) DIO mice treated with plant extract at 1 mg/kg body weight (bw), 10 mg/kg bw and 100 mg/kg bw

respectively. This treatment routine was continued for 28 d and the body weight and the physiological features were observed on a daily basis.

After 58 d the animals were sacrificed and histopathological studies, blood and lipid parameters/ profiles were tested and analysed.

Blood profiling:

A complete blood count which includes count of Red Blood Cells (RBC), eosinophils, lymphocytes, White Blood Cells (WBC), neutrophils, monocytes, total proteins, haemoglobin, and Thiobarbituric Acid Reactive Substance (TBARS) , lipid profile and blood glucose was carried out. The blood samples were collected by the tail nip method.

The total RBC count was estimated using the formula (Total RBC=N×dilution factor×volume correction factor) Where N=Total number of RBC cells counted in the chamber, Dilution=1: 200, Dilution factor=200. The WBC count was estimated by the formula (Total WBC count=N×50) N=Total number of WBC cells in 4 chambers i.e. 0.4 cu.mm of diluted blood. The total protein content in the serum was estimated using the Bradford method with Bovine Serum Albumin (BSA) as standard.

The total haemoglobin content was estimated using the High-Performance Liquid Chromatography (HPLC) method[18]. The total glucose content, triglycerides and cholesterol was estimated by using AGAPPE kits.

Histopathological studies:

Histopathological studies were performed to study the morphological features and to observe the changes in the islets of Langerhans cells (beta cells and acinar cells) in pancreas. The mice were euthanized with a high dose of anaesthetic agent[19] and a histopathological study of the pancreas was carried out. The specimen slides were prepared using the Hematoxylin and Eosin (H&E) staining method[20]. The images were captured using an inverted microscope.

Antioxidant assay:

The aqueous and methanolic extracts from native and *in vitro* propagated *E. bicolor* were evaluated for their antioxidant property by 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay as described by Brand *et al.*[21]. Different concentrations of the extracts ranging from 0.05 to 1.25 mg/ml were used in the study. The values obtained from results of our study on DIO mice were used in calculating the concentration of the aqueous extract. The calculations were carried out described by Earnest *et al.*[22]. Ascorbic acid and gallic acid solutions were considered as standard and positive control respectively. Respective solvent alone was considered as blank whereas the solvent with DPPH was used as negative control. All the experiments were carried out in triplicate. The absorbance was read at 517 nm. (Thermo Fisher: GENESYS 10S Ultra Violet (UV-Vis) v4.003 2L9Q082005 spectrophotometer). Percentage inhibition was calculated using the formula mentioned below. Percentage inhibition=((Absorbance control-Absorbance sample)/Absorbance control)×100. Percentage inhibition against concentration were plotted and half-maximal Inhibitory Concentration (IC_{50}) values were calculated using nonlinear regression.

RESULTS AND DISCUSSION

The emission maxima of alpha amylase was 342.5 nm, whereas alpha amylase with acarbose was at 347.5 nm. The emission spectra of native plant extracts with alpha amylase was 342 nm and that of *in vitro* plantlets was 347.5 nm (fig. 1). The results indicate that there is 1.5 % shift in absorbance in the case of *in vitro* plant extracts indicating increased binding affinity compared to native plant extracts.

Anti-diabetic activity of crude extracts of tissue culture and native plants on DIO mice including the C57BL/6J mice in group G1 to group G5 fed with carbohydrate rich diets for 30 d were obese and showed a significant increase in their body weight.

Complete blood count, lipid profile and blood glucose was carried out after 58 d (fig. 2). It was observed that the RBC count was similar in all the groups. Eosinophils and lymphocytes were significantly higher in the untreated mice compared to the mice treated with plant extracts and glibenclamide. The lymphocytes levels in mice across group G3 to G5 had no significant variations. A dose dependent reduction in the WBC levels in the mice across group 3 to group 5 was observed after the treatment period. The WBC levels were high in the DIO mice without any treatment and were significantly low in the DIO mice treated with glibenclamide. Similar results were observed with respect to neutrophil levels, monocyte levels, TBARS, total protein and hemoglobin levels. Similarly, the blood glucose and lipid profile showed a dose dependent reduction in mice treated

with aqueous extract of *in vitro* cultured plants across the group G3, G4 and G5 respectively (fig. 3). The blood glucose levels, free fatty acids, triglycerides and cholesterol were high in the group of untreated DIO mice whereas the DIO mice treated with glibenclamide had significantly reduced levels of blood glucose, free fatty acids, triglycerides and cholesterol. A dose dependent increase in the High-Density Lipoproteins (HDL) levels were observed in mice from group 3 to 5 groups. The HDL levels were low in the DIO mice without any treatment and the DIO mice treated with glibenclamide had higher levels of HDL.

H&E of the pancreatic tissue was carried out to study the histopathological differences amongst the six groups of mice taken (fig. 4). The DIO mice from group G1 showed considerable cell damage and degenerated acini cells compared to the DIO mice treated with glibenclamide. The DIO mice treated with plant extracts (G3-G5) showed a gradual increase in the number of beta cells and improved architecture of the acini cells over the control group (G1).

The percentage inhibition of methanolic and aqueous extracts from native plants were 83.21 % and 62.34 %, whereas from the *in vitro* culture plantlets was 72.38 % and 73.26 % respectively. The percentage inhibition *vs.* concentration for methanolic and aqueous extracts are shown in fig. 5 and fig. 6 respectively. The ascorbic acid (standard) had an IC_{50} value of 0.41 mg/ml. The IC_{50} values from methanolic and aqueous extract of native plants was 0.33 mg/ml and 0.62 mg/ml, whereas from the *in vitro* culture plants it was 0.73 mg/ml and 0.50 mg/ml respectively.

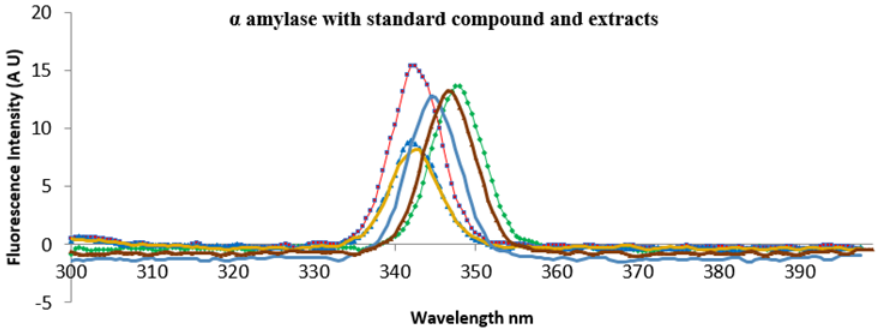

Fig. 1: alpha amylase assay with standard compound and *E. bicolor* extracts
Note: ( ): a; ( ): b; ( ): c; ( ): d; ( ): e; and ( ): f

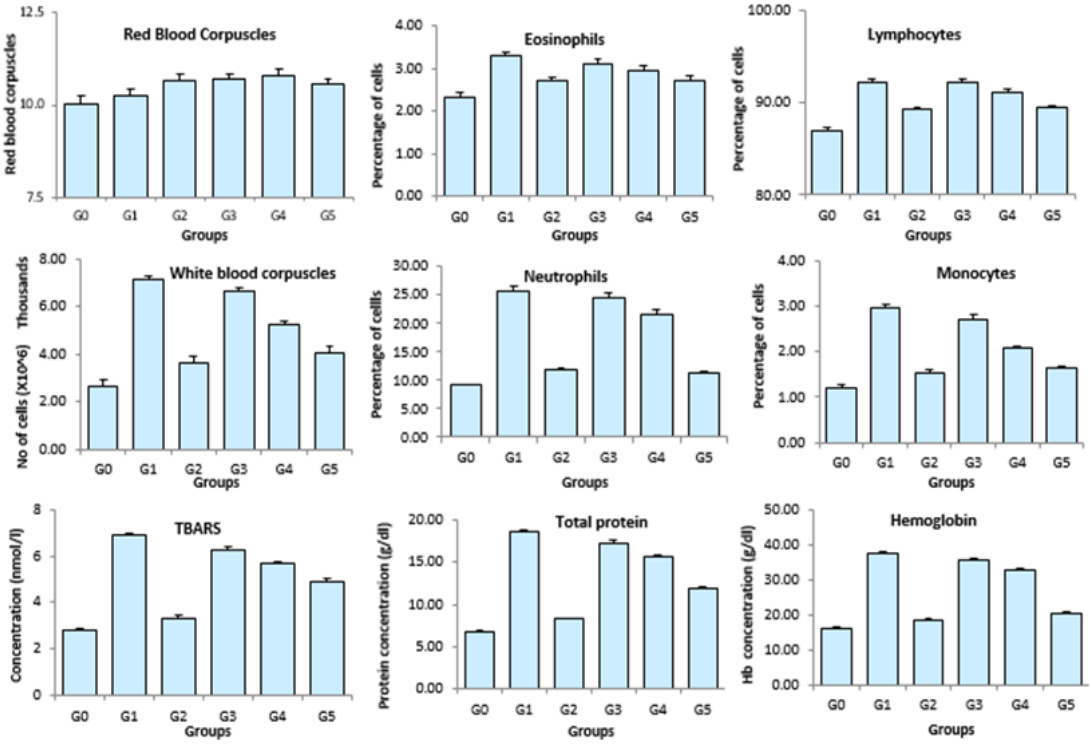

Fig. 2: Blood parameters of mice from group G0 to G5

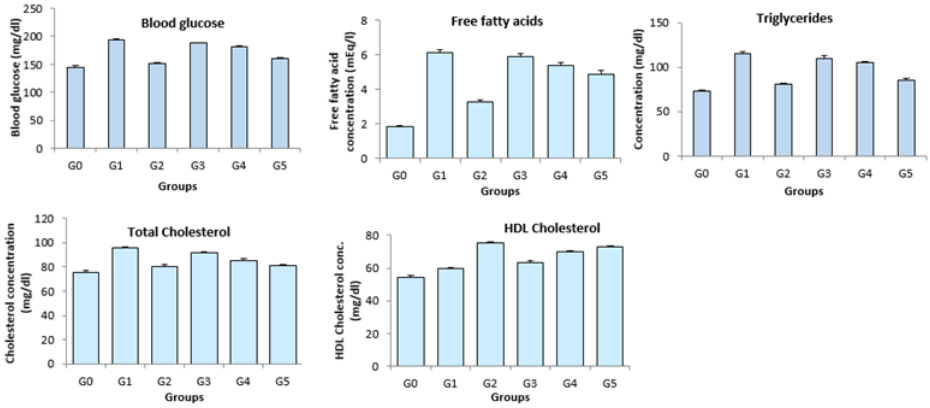

Fig. 3: Lipid parameters of mice from group G0 to G5

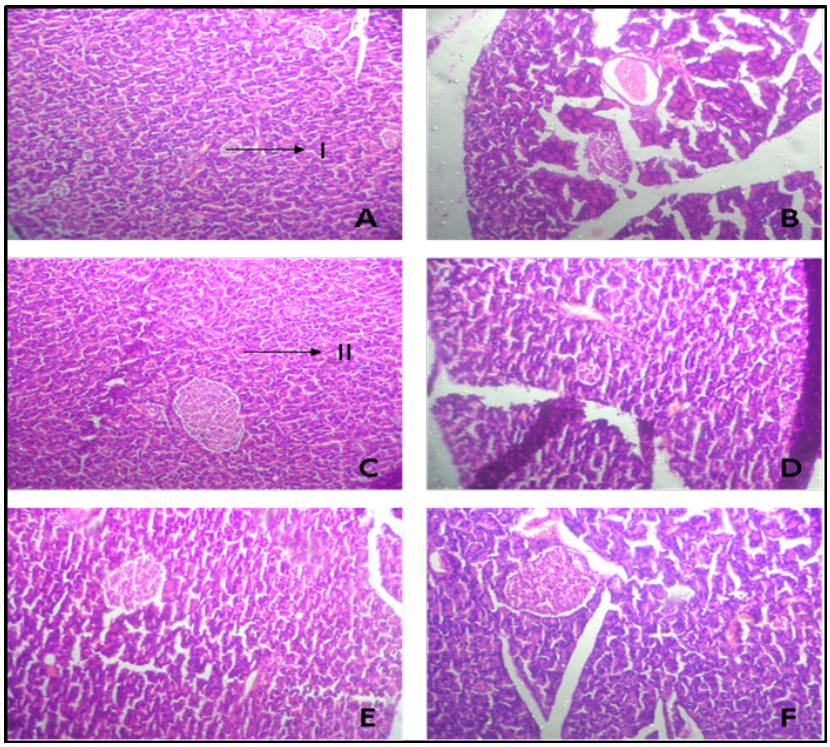

Fig. 4: Histopathologcal studies on pancreas of mice (groups G0 to G5)

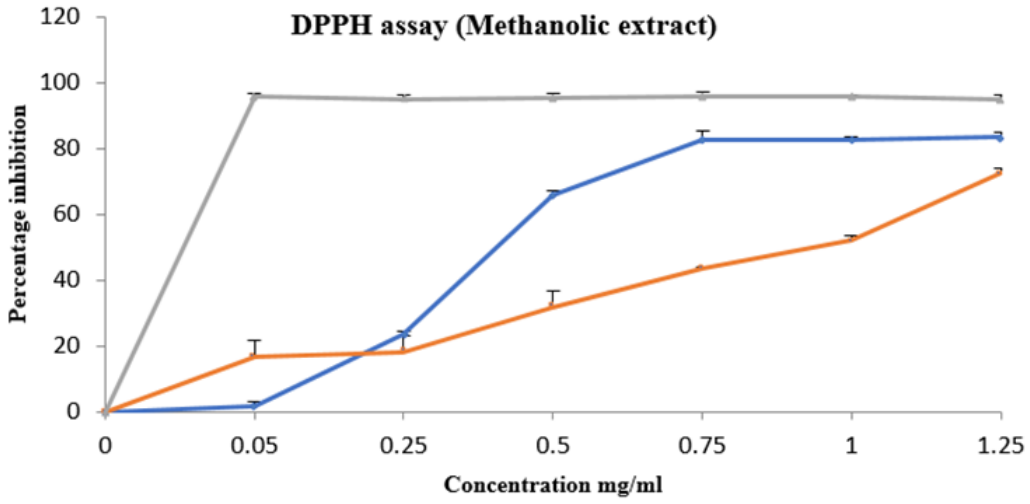

Fig. 5: DPPH assay of methanolic extracts from native and *in vitro* culture plants
Note: ( ): b; ( ): c and ( ): a

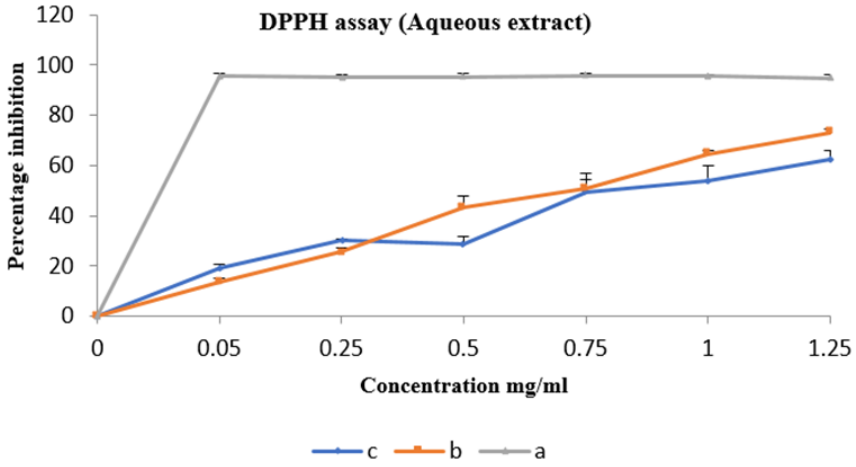

Fig. 6: DPPH assay of aqueous extracts from native and *in vitro* culture plants
Note: ( ): c; ( ): b and ( ): a

DIO mice have been used in many studies to evaluate the therapeutic properties of various plant extracts. The methanolic extract of *Salvia officinalis* was tested for its anti- diabetic properties by Kheder *et al.*[23]. The authors had administered the extracts to DIO mice at two doses of 100 and 400 mg/kg/ day for 5 w. They have observed improvement in glucose tolerance and decrease in insulin resistance that was similar to the effects produced by standard anti-diabetic drugs. Attakpa *et al.*[24] have reported using DIO mice groups to study the anti-diabetic effect of *Moringa oliferia* at three different concentrations (200, 400 and 600 mg/kg body weight of mice) and compared the results with glibenclamide treatment group. The authors have reported reduced blood glucose levels and an increased expression of Peroxisome Proliferator-Activated Receptor Alpha (PPARα) content (by western blotting) in DIO mice. Sunil *et al.*[25] have reported the anti-diabetic and anti-obesity effect of *Memeceylon umbellatum* using DIO mice. The authors have performed the histological examinations and analysed the serum parameters and the gene expression patterns. They have observed a significant reduction in the body weight, fasting glucose and triglyceride levels of mice treated with 250 mg/kg body weight of extracts. They have mentioned an improvement in glucose tolerance and reduced levels of serum Interleukin-6 (IL-6) and Low-Density Lipoprotein (LDL) levels along with down regulation of IL-6, Plasminogen Activator Inhibitor-1 (PAI-1) and Apolipoprotein B (ApoB) gene from the gene expression studies. Ashwini *et al.*[26] has reported testing the anti-inflammatory effects of *E. bicolor* plant extracts on Swiss albino mice. They reported a dose dependent reduction in edema of paws. However, there have not been any reports of using DIO mice as animal models to study the anti-diabetic properties of *E. bicolor* plant extracts. In this study, DIO mice were treated with aqueous extracts of *E. bicolor* plant extracts at three different concentrations. The main factors in the onset of diabetes viz., blood glucose, body weight, triglycerides and total cholesterol levels were reduced in a dose dependent manner in the mice treated with plant extracts. Our results correlate with the reported literature validating the anti-diabetic potential in *E. bicolor* plants and to the best of our knowledge is a first time report.

Upon analysing the IC_{50} values from the results, it was observed that the tissue culture plants had better antioxidant property than the native plants. Ashwini *et al.*[9] have observed a maximum inhibition percentage of 93.58 % and an IC50 value of 14.84 µg/ml from the crude methanolic extract of *E. bicolor* whereas Sharad *et al.*[27] have reported the antioxidant property from the methanol and hexane extracts of *E. bicolor* to be modest. In our present study aqueous extracts of *E. bicolor* was used in this study, it was safe and non-toxic on the model organisms. The effective dosage was identified and used to validate the antioxidant property of the plant extracts of native and tissue engineered plantlets. Sharad *et al.*[27] have investigated the Absorption, Distribution, Metabolism, and Excretion (ADME) properties of 32 phytochemicals using SwissADME and have identified two molecules i.e. phthalic acid butyl dodecyl ester and pyrimidine-2,4,6 (1H,3H,5H)-trione-5-(3-(2-(4-tertbutylphenoxy) ethoxy) benzylidene as possible drug candidates. The authors have performed antimicrobial assays using crude extracts of *E. bicolor* and have reported prominent antibacterial property against *Bacillus pumilus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and moderate antifungal property towards *Aspergillus niger* and *Aspergillus*

fumigatus, they have also reported moderate cytotoxic activity in reducing the cell viability of Michigan Cancer Foundation-7 (MCF-7) breast cancer cell lines.

Further a preliminary screening on the binding affinity of the plant extracts to alpha amylase was also performed. The present research investigation represents the first reports on antioxidant and amylase assays on the tissue cultured plantlets of *E. bicolor* and compared pharmacologically with the native plant extracts.

The plant extracts of *E. bicolor* have scientifically proven to contain anti-diabetic properties, hence there is a need to conserve and utilize its medicinal properties to the fullest. The current research has led to the development of a rapid and efficient *in vitro* propagation technique for the endemic medicinal plant *E. bicolor* Roxb. Testing of the *in vitro* generated plantlet extracts on model organisms (DIO mice) has shown positive results indicating the presence of anti-diabetic and antioxidant properties. *In vitro* generated plantlet extracts showed better activity when compared to native plant *Exacum* crude extracts. The present study not only bioengineered the plant through *in vitro* propagation, but also gave insights to the bioactive metabolites that can be served as lead molecules for treating type II diabetes.

Acknowledgments:

The authors were thankful to Skanda Life Sciences Pvt. Ltd. Bengaluru for helping with the animal models and histopathological studies. The authors also thank Technical Education Quality Improvement Programme (TEQIP-2 and TEQIP-3) for providing the financial assistance during this research work.

Conflict of interests:

The authors declared no conflict of interests.

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