Antidiabetic and *in vitro* antioxidant potential of *Hybanthus enneaspermus* (Linn) F. Muell in streptozotocin–induced diabetic rats

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ABSTRACT

Objective: To evaluate antidiabetic and antioxidant potential of *Hybanthus enneaspermus* in different models. **Methods:** The oral glucose tolerance test (OGTT) and normoglycemic effect of alcoholic extract of *Hybanthus enneaspermus* (AHE) were evaluated at a dose of 125, 250 and 500 mg/kg *p.o.* while hypoglycemic activity and effect on body weight were tested at 250 and 500 mg/kg *p.o.* per day for 21 days in Streptozotocin (STZ) induced diabetic rats. Further, glucose uptake by hemidiaphram was also evaluated. The total polyphenolic and flavonoid were determined and their correlation with various antioxidant assays was also determined. **Results:** The results showed high level of phenolic content in AHE. AHE also exhibited higher total antioxidant capacity, good reducing power and a significant scavenger of reactive oxygen species like DPPH radical, nitric oxide, hydrogen peroxide and deoxyribose. Furthermore there was a significant increase in the body weight and decrease in the blood glucose level on treatment with the AHE. AHE increased glucose uptake on isolated rat hemi−diaphragm compared to control group. **Conclusions:** AHE reduce blood glucose level in STZ−induced diabetic model. It does not show significant effect in normoglycemic study but showes significant effect in OGT. AHE has significant antioxidant activity, which may be attributed to high phenolic content.

1. Introduction

Diabetes mellitus, one of the most common endocrine metabolic disorders has caused significant morbidity and mortality due to microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (heart attack, stroke and peripheral vascular disease) complications[1]. This is mainly attributed to the rapid rise in unhealthy life style, urbanization and aging. The number of diabetics increases according to World Health Organizations (WHO) approximation[2]. In diabetes, hyperglycaemia generates reactive oxygen species (ROS) which in turn cause lipid peroxidation and membrane damage and thus, plays an important role in the production of secondary complications in diabetes mellitus such as kidney, eye, blood vessel, and nerve damage. Antioxidants have been shown to prevent the destruction of β−cells by inhibiting the peroxidation chain reaction and thus they may provide protection against the development of diabetes. Plants containing natural antioxidants (tannins, flavonoids, vitamins C and E) can preserve β−cell function and prevent diabetes induced ROS formation[3]. Polyphenols, which are classified into many groups such as flavonoids, tannins, stilbenes with known health−beneficial properties, which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes, anti−inflammatory action and antidiabetogenic potentiality[4].

Hybanthus enneaspermus (*H. enneaspermus*) Muell belongs to family Violaceae. It is an herb or a shrub distributed in the tropical and subtropical regions of the world. The plant is used as an aphrodisiac, demulcent, tonic, diuretic, anti−convulsant and antimalarial and used to treat urinary infections, diarrhoea, leucorrhoea, dysuria, inflammation and male sterility[5,6]. The plant is used by some tribe in Orissa to cure diabetes[7]. In Ayurvedic literature, the plant is reported to cure conditions of "Kapha" and "Pitta", urinary calculi, strangury, painful dysentery, vomiting, burning sensation, wandering of the mind, urethral discharge, blood trouble, asthma, epilepsy, cough, and to give tone to the breasts[8]. Pharmacologically the plant is reported to possess antimicrobial, anti−inflammatory, antitussive, antiplasmodial, anticonvulsant and free radical

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scavenging activities[9]. Even though the plant is used traditionally for the treatment of diabetes, scientific evidence is still scare. Therefore this investigation was conducted to scientifically validate its traditional use.

2. Materials and methods

2.1. Chemicals and instruments

1, 1–diphenyl–2–picryl–hydrazil (DPPH) and streptozotocin (STZ) were purchased from Sigma Aldrich while glucose estimation kit was purchased from Span diagnostics, Mumbai, India. All other chemicals and solvents used were of analytical grade. The absorbance measurements were recorded using the ultraviolet–visible spectrophotometer (Shimadzu, Pharmaspec–1700).

2.2. Plant material and preparation of the extract

The plant material was procured from herbal vendor at Chennai, and identified by the chief botanist Tampcol, Anna Hospital Chennai. A voucher specimen (COG/HE/01/08) was retained in the Herbarium of Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi for future reference. The dried powder of whole plant material (1 kg) was extracted in a Soxhlet apparatus by ethanol. The solvent was removed from the extract in a rotary evaporator and dried.

2.3. Phytochemical standardization

In order to establish a relationship between the chemical content and the antidiabetic activity, the total phenol, flavonoid and flavonol contents of alcoholic extract of *H. enneaspermus* (AHE) were determined. Preliminary phytochemical composition of AHE was analyzed for their chemical constituents according to the official method[10, 11]. Total phenolic (TP) content of AHE was determined using Folin–Ciocalteu method while total flavonoid and total flavonols content was determined using rutin as the standard according to the method of Kumaran & Karunakaran, 2006[12,13].

2.4. Antioxidant assays

Due to the complex nature of plant extract and the diverse mechanism of protective effects, antioxidant activity evaluation by any single method seems to be rather unrealistic[14]. Therefore antioxidant activity was evaluated by different methods. All readings were performed in triplicates and the percentage inhibitions were calculated in the form of IC_{50} value.

2.4.1. DPPH radical scavenging activity

The antioxidant activity of AHE was evaluated on the basis of the radical scavenging effect of the stable DPPH free radical[15]. Ascorbic acid was used as a standard compound in DPPH assay and absorbance was at 517 nm.

2.4.2. Nitric oxide scavenging activity

Griess Illosvoy reaction is generally used to determine the nitrite ions, which is produced by sodium nitroprusside in aqueous solution at physiological pH by interaction with nitric oxide, and oxygen. The absorbance was measured at 546 nm and IC_{50} was calculated by using rutin as control[16].

2.4.3. Hydrogen peroxide scavenging activity

The scavenging activity of AHE was evaluated by method described by Jayaprakasha *et al*, and absorbance of final solution was measured at 230 nm with rutin as a positive control[17].

2.4.4. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging of AHE was evaluated by deoxyribose method and absorbance was measured at 532 nm against a blank with butylated hydroxy anisole (BHA) as a positive control[18].

2.4.5. Total antioxidant capacity

Phosphomolybdenum method as described by Prieto *et al* was used to determine the total antioxidant capacity of the AHE where absorbance was measured at 695 nm against the blank. The antioxidant activity was expressed as the number of equivalent of ascorbic acid[19].

2.4.6. Assay of reducing power

Potassium ferricynide method was used to determine the reducing power of AHE. Absorbance was measured at 700 nm and higher absorbance indicates stronger reducing power[20].

2.5. Pharmacological evaluation

2.5.1. Experimental animals

Male albino wistar rats (100–140 g) were used for this study. They were housed in polypropylene cages under standard laboratory conditions [12 h light/12 h darkness, (21±2) ℃]. The animals were given standard pellets diet (Mona laboratoty animal feed) and water *ad libitum* throughout the experimental period. The experimental study was approved by the Institutional Animal Ethical Committee of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

2.5.2. Preparation of the test samples

Extract as well as drug was suspended in 0.5% carboxymethylcellulose (CMC) in distilled water prior to oral administration to the experimental animals.

2.5.3. Acute toxicity study

Acute toxicity study was carried out for the AHE following OECD 425 guidelines. AHE was suspended in 0.5% w/v CMC and was given at a dose of up to 5 g/kg *p.o.* body weight to overnight–fasted, healthy rats (*n* = 3). The animals were observed continuously for behavioral changes like abnormal

locomotion, respiratory distress, and uncoordinated muscle movements for 24 h.

2.5.4. Oral glucose tolerance test in normal rats (OGTT)

Rats were divided into five groups (n=6) and were administered with 10 mg/kg glibenclamide and dose of 125, 250, 500 mg/kg per oral of AHE. Glucose solution 2 g/kg was administered 30 min after the administration of the extract. Blood samples were withdrawn from retro–orbital at intervals of 30, 60 and 120 min of glucose administration[21].

2.5.5. Effect of AHE in normoglycemic rats (NG)

Animals in the control group received CMC solution (orally). The animal of test groups were treated with the AHE at the dose of 125, 250 and 500 mg/kg in oral. Blood samples were collected at 30, 60 and 120 min, after the drug administration[21].

2.6. Evaluation of antidiabetic activity

2.6.1. Induction of diabetes

Diabetes was induced in overnight–fasted rats by a single intraperitoneal injection of freshly prepared STZ (65 mg/kg, dissolved in 0.1 M cold citrate buffer, pH 4.5). STZ injected animals were given 5% glucose solution for 24 h following streptozotocin injection for prevention from initial drug–induced hypoglycemic mortality. After 72 h, the blood was withdrawn by retro–orbital puncture under light ether anesthesia and the blood glucose level was estimated. After 1 week of induction, blood glucose level was estimated again and a fasting blood glucose level of more than 200 mg/dL was considered as diabetic and was enrolled in the study[22].

2.6.2. Chronic treatment model

Diabetic rats were divided into 5 groups and were daily treated from days 1 to 21 as follows: 2 groups received AHE at the doses of 250 and 500 mg/kg, respectively; group 3 received glibenclamide (10 mg/kg); group 4 were untreated diabetic control (DC) rats; group 5 were the normal control (NC) group and received vehicle. The blood glucose level was determined in fasted rats before administration of drugs (day 1) and at weekly interval by retro orbital puncture under light ether anesthesia. The plasma glucose estimation was done by the glucose oxidase/peroxidase (GOD/POD) method using a standard kit obtained from Span Diagnostics, India. Body weight of all experimental animals was recorded using a digital weighing scale[21].

2.7. In vitro study

2.7.1. Effect of AHE on glucose utilization by isolated rat hemi diaphragm

Glucose utilization by rat hemi–diaphragm was estimated by the method described by Hemalatha *et al*. Regular insulin (Biocon Ltd.) was used as a positive control group and albino rats diaphragms were used in this assay. Glucose uptake per gram of tissue was calculated as the difference between the initial and final glucose content in the incubated medium[23].

2.8. Statistical analysis

All the values of the experimental results were expressed as mean±standard error of mean (SEM). Two–ways ANOVA followed by Bonferroni post test was performed for evaluation of OGTT, Normoglycemic study and blood glucose of STZ induced diabetic rats. One–way ANOVA followed by Tukey´s Multiple Comparison test was applied for the statistical analysis of body weight. For all other analysis unpaired t–test was applied to show the statically significance. GraphPad Prism (version 4) software was used for all statistical analysis, and $P<0.05$ was considered as significance.

3. Results

3.1. Phytochemical standardization

The qualitative phytochemical screening of the AHE revealed the presence of flavonoids, terpenes, phenols, anthraquinones, glycosides, polyoses, alkaloids, saponins, tannins. From the observation, it was found that the content of phenolics and flavonoids were high in ethanolic extracts but flavonols were comparatively less. The total amount of phenolic was 71.95 mg/gm equivalent to gallic acid and the total flavonoid and flavonol contents were 20 mg/g and 0.68 mg/g with respect to rutin.

3.2. Acute toxicity study

No toxic effects were observed at a dose of 5 g/kg body weight. Hence, there were no lethal effects in any of the groups.

3.3. Antioxidant activity

The free radical scavenging activity (DPPH) of AHE was studied by its ability to reduce the DPPH, and found to have considerable free radical scavenging activity as indicated by their IC_{50} values [(164.52±1.48) μg/mL] in comparison to ascorbic acid [(77.92±1.93) μg/mL] (Figure 1). Nitric oxide is a very unstable species and reacts with oxygen molecule to produce stable nitrate which can be estimated by using Griess reagent. AHE had significance nitric oxide scavenging activity [IC_{50} value (211.47±3.08) μg/mL] comparing with standard rutin [IC_{50} value (98.06±6.10) μg/mL] (Figure 2). Hydrogen peroxide, although not a radical species plays a role in oxidative stress. AHE showed good activity in depleting H_2O_2, with an IC_{50} value of [(190.74±6.26) μg/mL] comparing with rutin [(87.07±0.52) μg/mL] (Figure 3). The effect of AHE on inhibition of hydroxyl radical production was assessed by the iron (II)–dependent deoxyribose damage assay. AHE showed significance hydroxyl radical scavenging effect with an IC_{50} value of (255.83±6.93) μg/mL

in comparison to standard BHA [(123.55±1.62) μ g/mL](Figure 3). The total antioxidant capacity of AHE was determined by the linear regression equation of the calibration curve (y = 0.005x+0.042, r^2= 0.996) and was expressed as the number to equivalent of ascorbic acid [(77.92±1.93) μ g/mL plant extract]. It was (42.31±0.65) μ g/mL. The AHE exhibited significant reducing power comparing to standard ascorbic acid.
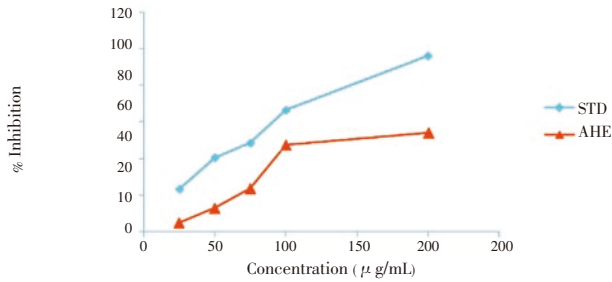

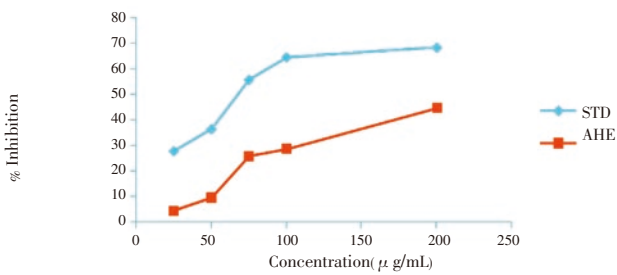**Figure 4.** Anti−oxidant scavenging activity of AHE in hydroxyl radical scavenging assay.

Figure 1. Anti−oxidant scavenging activity of AHE in DPPH scavenging assay.
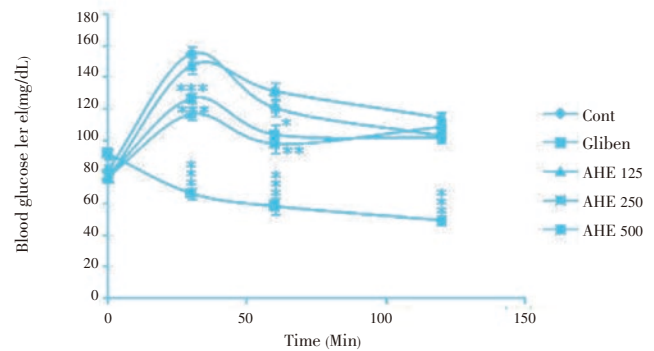
Figure 5. Effect of AHE in OGTT.
*P<0.05, **P< 0.01, ***P<0.001, company with normal control.

Figure 2. Antioxidant scavenging activity of AHE in nitric oxid scavenging assay.
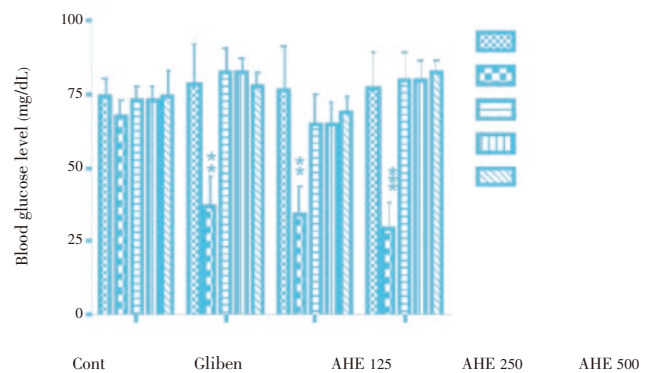
Figure 6. Effect of AHE in normoglycemic study.
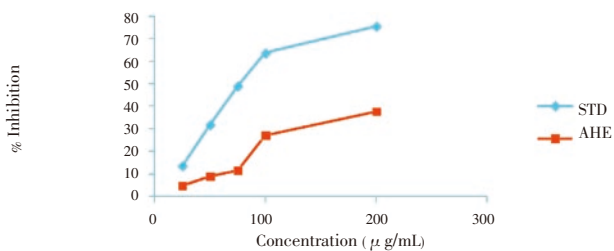*P<0.05, **P< 0.01, ***P<0.001, company with normal control.

Figure 3. Anti−oxidant scavenging activity of AHE in H_2O_2 scavenging assay.

3.4. Co−relationship between the total antioxidant capacity and the total phenolic content

The correlation was done by calculating the r^2 (Correlation coefficient) which was obtained by plotting the total antioxidant activities of AHE versus its total phenolic content. It showed linear correlation with a value of 0.972.
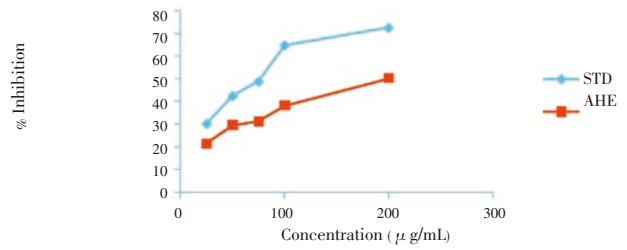
3.5. Hypoglycemic effect of ethanolic extract

The results clearly indicated that the blood glucose level of diabetic control group increased significantly comparing with normal group, which revealed that diabetic animal mode was successfully established. AHE showed significant hypoglycemic effect from 7th day onwards to 21st day of treatment. It was also observed that there was no significance difference in AHE treated and glibenclamide treated group

Table 1
Effect of AHE on serum glucose level in streptozotocin induced diabetic rats.

Group	Fasting plasma glucose concentration (mg/dL)			
	1st day	7th day	14th day	21st day
Normal control	92.36±3.47	85.37±3.42	80.95±1.75	88.75±2.69
Diabetic control	314.51±19.87[a]	320.12±15.98[a]	307.36±17.56[a]	302.82±23.54[a]
Glibenclamide (10 mg/kg)	360.19±13.68[a]	237.03±12.95[ab]	213.93±4.71[ab]	1 56.95±5.03[ab]
Ethanol extract (250 mg/kg)	300.58±17.54[a]	260.26±17.70[ab]	242.56±15.46[ab]	231.41±19.5[abc]
Ethanol extract (500 mg/kg)	301.92±14.03[a]	269.40±12.94[ab]	254.81±11.15[ab]	232.64±12.53[abc]

Values are mean±SEM of 6 animals in each group. a: *P*<0.05 comparing with the normal control; b: *P*<0.05 comparing with the diabetic control; c: *P*<0.05 comparing with glibenclamide treated group and d: *P*<0.05 comparing with the AHE extract treated group (Two–way ANOVA followed by Bonferroni post test).

Table 2
Effect of AHE on body weight in streptozotocin induced diabetic rats.

S. No	Body weight in STZ–induced diabetic rats				
	Normal control	Diabetic control	Glibenclamide	250 mg/kg	500 mg/kg
0th day	119.66±1.76	124.83±1.01	122.80±2.35	121.33±1.52	116.83±2.73
21st day	128.66±1.33	103.50±4.62[a]	127.66±4.52[b]	125.66±3.56[b]	119.33±3.44[b]

Values are mean±SEM of 6 animals in each group. a: *P*<0.05 comparing with normal control; b: *P*<0.05 comparing with diabetic control (One–way ANOVA followed by Tukey ́s Multiple Comparison test).

Table 3
Effect of AHE on glucose utilization by hemi–diaphragm.

Group	Incubation medium	Glucose uptake (mg/g/30 min)
Control	Tyrode solution with glucose (2%)	18.11±1.12
Insulin	Tyrode solution with glucose (2%) + Insulin (0.25 IU/mL)	27.55±0.49[a]
Insulin + plant extract	Tyrode solution with glucose (2%) + Insulin (0.25 IU/mL)+ plant extract (25 mg/mL)	24.07±0.78[a]
Plant extract	Tyrode solution with glucose (2%) + plant extract (25 mg/mL)	22.93±0.45[a]

a: *P*<0.05 comparing with control group, results were analyzed by unpaired *t*–test.

up to 14th day of treatment, but, on 21st day, glibenclamide treated group showed more significancant hypoglycemic effect than AHE treated group. Further, the result also showed that there was no dose dependant reduction of blood glucose levels in AHE treated groups (Table 1). OGTT study showed that rats treated with 500 mg/kg of AHE exhibited high tolerability of glucose after 30 minutes. However, there was no significant effect on normoglycemic rats (Figure 5, 6). The body weight of normal rats, AHE (250 and 500 mg/kg) and standard drug treated groups increased significantly comparing with diabetic control group (Table 2).

3.6. Effect of AHE on glucose utilization by hemidiaphragm

Hemi–diaphragm showed that AHE possess significant glucose uptake when compared with control group (Table 3).

4. Discussion

Several studies have demonstrated the beneficial effects of *H. enneaspermus*. Based upon the ethnopharmacological reports of the treatment of diabetes with this plant, the present study was carried out to determine the protective effects of AHE on diabetic complications and thus, to validating its traditional use[7]. Further AHE showed significance antioxidant potential in *in vitro* model. The

relationship between phenolic, flavonoid, and total flavonol content were also investigated. To the best of our knowledge, this is the first report on the antidiabetic effect of AHE.

It is not always easy to determine if the consuming plant materials are always safe. In fact there are a huge number of plants with a wide range of toxicity. Our result reveals that there was no lethality or any toxic reactions at the selected dose.

In normal and glucose loaded rats, the AHE showed improvement in glucose tolerance. However it was less significant than glibenclamide treated rats. The observation showed that AHE reduced blood sugar level in diabetic rats which was similar to glibenclamide, except on day 21st. AHE also significantly decreased the blood glucose level of glucose loaded rats. A dose dependent hypoglycemic effect of AHE was observed in normal rats up to 500 mg/ kg. However, the response decreased at higher dose *i.e.* 500 mg/kg in the diabetic model. Such a phenomenon of less hypoglycemic response at higher doses was also observed in some indigenous plants like *Aegle marmelose*, *Murraya koenigii*, and *Vinca rosea*[24]. It is reported that flavonoid–rich extract results in the significant reduction of peak level of sugar within 2 h time thus strengthens the antidiabetogenic potentiality of AHE[25].

In our study STZ was used to induce type 1 diabetes mellitus in rats since the mechanism is very similar to that of the hyperglycemic nonketotic diabetes mellitus in human. STZ causes selective damage to pancreatic β –cell

through the release of nitric oxide, and the action of STZ on mitochondria generates SOD anions, which leads to diabetic complications[26]. Loss of body weight is due to increased muscle destruction and loss of proteins contents in the tissue[21]. Diabetic rats treated with the AHE showed an improvement in body weight in comparison to the diabetic control and standard glibenclamide treated group, which signifies its protective effect in controlling muscle wasting i.e. reversal of gluconeogenesis. Moreover, the ability to protect body weight loss seems to be as a result of its ability to reduce hyperglycemia. The *in vitro* study on glucose uptake by hemidiaphragm suggested that AHE probably has no direct insulin–like effect, which can enhance the peripheral utilization of glucose.

The ethanolic extracts have been chosen because of its expected flavonoid contents that were reported to have antidiabetic activity. Eventually a good relationship was also observed between phenolics and antioxidant activity which further confirms this view[27]. Nitric oxide scavenging activity depends upon the concentration of the extract plant. It shows this scavenging activity is useful for the prevention from disease in various types caused by excessive NO generation in the human body[28]. Hydroxyl radical is highly reactive in nature. It is capable to damage a vast number of biological molecules in the human body, and to join nucleotides in DNA which cause carcinogenesis, mutagenesis, and cytotoxicity[29]. In addition, the good correlation between antioxidant activity and reducing power in some plant extracts has been observed[30]. Therefore, the reducing capacity may be used as an indicator of the potential antioxidant activity. Hydrogen peroxide itself is not very reactive in nature, but sometimes it can produce hydroxyl radical which might be harmful to the biological system[31]. Therefore, abolition of H_2O_2 is needed to protect body as well as food systems. Polyphenols, found in the dietary source, can protect mammalian and bacterial cells from cytotoxicity induced by H_2O_2[32]. Therefore, the phenolic compound of the AHE is involved in removing the H_2O_2. Extract has shown significance antioxidant activity against these radicals, so they can protect pancreases as well as body from these radicals and play an important role in the diabetic study.

The phytochemical analysis of AHE showed the presence of tannins, flavanoids, saponins and sterols. Their antidiabetic ability to regenerate the pancreatic β –cell has already been justified[33]. Sterols can decrease blood sugar in experimental animal models[34]. The antioxidant activity of the phenolic, tannins, flavonoid compounds which are very common both in edible and inedible plants, are attributed to its redox properties. With these properties they can act as reducing agents, hydrogen donators and singlet oxygen quenchers. They also have metal chelating properties[35]. Polyphenolic scavenging action is mainly due to their hydroxyl groups. They are very important plant constituents which can protect body from different type of oxidative stress[36–39]. Flavones glycosides have also been reported as strong antioxidants and potent hypoglycemic agents.

In conclusion, the present study reveals that AHE has significance antioxidant activity and can decrease glucose level significantly in OGTT in a dose dependent manner. The drug also shows significant hypoglycemic activity but the effect is dose independent. The rat hemidiaphragm study displayed significant uptake of glucose. Although the antidiabetic activity of AHE is justified, but further investigation is still in progress to validate its action mechanism.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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