ABSTRACT
Enicostemma littorale (Family Gentianaceae) is one of the plants among a rich heritage of indigenous medicinal plants of India which has been empirically used by the tribal inhabitants for the treatment of diabetes and it is present in most of the polyherbal antidiabetic formulations available in our country. Some of the studies have indicated its hypoglycemic activities. However, systematic experimental studies in diabetic rats and clinical studies have not been reported to our knowledge. In the present investigation, we have studied the antidiabetic activity of aqueous extract of E. littorale. To prepare the aqueous extract, dried whole plant of E. littorale was heated with water for about 72 hrs at 70\(^0\)-80\(^0\) C, intermittently changing water and adding fresh water. The filtrate was concentrated at 80\(^0\)-90\(^0\) C until an aqueous extract containing 3.75 mg of E. littorale/ml was obtained. This aqueous extract was used for the animal experiments.

Healthy adult female albino Wistar rats were given a single tail vein injection 45 mg/kg of streptozotocin (STZ) to produce Type 1 i.e. Insulin-Dependent Diabetes Mellitus (IDDM). To induce NIDDM, STZ (70 mg/kg) was administered intraperitoneally, to five-day old pups of albino Wister rats having free access to food and water, weaned on 23\(^{rd}\) day and then allowed to grow further. After 12 weeks, the animals showing fasting glucose levels (\(\geq 140\) mg/dl), were considered as NIDDM model. All the rats were housed under standard conditions with diet and water provided ad libitum throughout the study period. They were randomly divided into six different groups: Control, Control treated, IDDM, IDDM treated, NIDDM and NIDDM treated. The animals were given the aqueous extract of E. littorale (500 mg/kg) p.o. daily for six weeks. During the six weeks study period, changes in the body weight, water intake and food intake were recorded in all groups of the animals. At the end of six weeks, blood samples were collected from the retino-orbital plexus of eye; serum (separated by centrifuging) was analysed for glucose, creatinine, Blood-Urea-Nitrogen (BUN), cholesterol and triglycerides. Serum insulin was measured by radioimmunoassay method using kits from Board of Radioactivity and Isotope Technology (BRIT, Mumbai). We also studied the effects of aqueous extract of E. littorale on Oral
Glucose Tolerance Test (OGTT) and Intravenous Insulin Tolerance Test (ITT). OGTT, AUC\textsubscript{glucose}, AUC\textsubscript{insulin} and $K_{\text{ITT}}$ were calculated to evaluate the effect of *E. littorale* on insulin resistance.

IDDM animals showed various cardinal symptoms of diabetes such as, significant loss of body weight, polyphagia, polyuria and polydipsia. *E. littorale* treatment significantly prevented the loss in body weight, polydipsia and polyphagia in these IDDM rats. NIDDM rats were found to have a significant decrease in body weight, which was prevented by *E. littorale*. However, the gain in weight by NIDDM rats was found to be significantly less as compared to the gain in weight by control rats. In contrast to IDDM, NIDDM rats were having almost same food and water intakes as those of the Wistar control rats and further, these values remained unaffected by the *E. littorale* treatment. Clinical symptoms of diabetes (polyuria, polydipsia and polyphagia) were more prominent among IDDM rats and *E. littorale* prevented these alterations.

IDDM rats showed hyperglycaemia, hypoinsulinaemia and higher AUC\textsubscript{glucose}. *E. littorale* treatment significantly decreased both the serum glucose levels and AUC\textsubscript{glucose} in these animals. IDDM rats were hypoinsulinemic and $K_{\text{ITT}}$ was not significantly different from the Wistar control. Both the serum insulin and $K_{\text{ITT}}$ remained unaffected by the treatment with *E. littorale* in IDDM rats. IDDM rats had significantly lower AUC\textsubscript{insulin} and the treatment with *E. littorale* produced an increase in AUC\textsubscript{insulin} as compared to the initial value. However, the AUC\textsubscript{insulin} after treatment with *E. littorale* remained less than that of the control animals.

NIDDM rats showed significantly elevated fasting as well as fed glucose and insulin levels. Treatment with *E. littorale* not only decreased glucose levels but also the raised insulin levels. Significantly higher AUC\textsubscript{glucose} as well as AUC\textsubscript{insulin}, and lower $K_{\text{ITT}}$ were the characteristics of NIDDM rats, suggesting these animals to be insulin resistant. Treatment with
E. littorale prevented significantly AUC_{glucose} and AUC_{insulin} of NIDDM rats. Even decreased K_{ITT} was prevented by the treatment with E. littorale.

IDDM rats had increased levels of serum cholesterol, which were significantly prevented by E. littorale. Serum cholesterol levels, of NIDDM rats, were not significantly different from the control rats and E. littorale did not alter these levels. Both IDDM and NIDDM rats showed high levels of serum triglycerides, which were significantly reduced by E. littorale.

We also found significant increase in serum creatinine, serum urea and BUN levels in IDDM and NIDDM rats. Treatment with E. littorale significantly prevented the raise in creatinine, urea and BUN levels. The serum creatinine of both IDDM and NIDDM animals after treatment with E. littorale was comparable with the control rats. Thus, E. littorale provides significant nephroprotection among diabetic rats.

The results of animal studies suggest that E. littorale is a potential herbal antidiabetic medication having remarkable insulin sensitizing action. It provides nephroprotection and normalizes the disturbed lipid profile among diabetic rats.

Based on the encouraging observations with E. littorale in animal studies, we extrapolated the results to the clinical settings. The aqueous extract (3.75 mg/ml), used for the animal study, was further processed. Water was evaporated from the aqueous extract of E. littorale, until it was reduced to a semisolid mass. Ghanvatis, (an ayurvedic form of pills, formulated as per Ayurvedic Pharmacopoeia) were prepared from that mass using pill making machine. Each ghanvati represented 250 mg of the dry weight of E. littorale.

We conducted an open, multicentric clinical study based on parallel group design. The Local Ethical Committee approved the protocol and proforma. 234 patients of either sex who attended various Ayurvedic hospitals (located in different parts of Gujarat) and medical camps during the period of Jan 98 to
June 99 were screened. After considering the protocol for inclusion and exclusion criteria, total 158 Type 2 diabetic patients were registered for the present clinical study. 45 nondiabetic healthy subjects who served as control were also selected and enrolled during random screening in the clinics and medical camps. The selected patients and nondiabetic subjects were explained about the procedures and written informed consents were taken from them. Detailed interviews were performed with the help of a pre-tested and validated carefully planned questionnaire and the clinical history sheet was filled separately for every patient and every nondiabetic subject. Height, weight, waist and hip girths were measured; WHR as well as BMI were calculated for each person. All these patients were given 4 ghanvatis of \textit{E. littorale} (250 mg \textit{E. littorale} per ghanvati) two times a day (b.i.d.) for a period of nine months. Nondiabetic subjects were not given the treatment of ghanvatis. Before beginning the treatment with \textit{E. littorale}, blood pressures and pulse rates were measured. Urine sugar was estimated by using Diastix reagent strips. 2-ml blood was collected from the cubital veins of the patients and blood glucose was measured using Glucostix reagent strips with the help of glucometer. The remaining blood was centrifuged, and separated serum was analyzed for creatinine, cholesterol, HDL-cholesterol and triglycerides. Serum insulin was measured by radioimmunoassay method. Estimation of all these parameters were repeated afterwards at the interval of 1\textsuperscript{st}, 3\textsuperscript{rd}, 6\textsuperscript{th} and 9\textsuperscript{th} month of active treatment with \textit{E. littorale}.

An overweighed and obese person is more prone to develop insulin resistance. BMI and WHR of 61\% diabetic males and 75\% of diabetic females in our present study were found significantly higher in the present study indicating that insulin resistance is more prevalent among Indian type 2 diabetic patients.

Among nondiabetics, 56\% were having medium type of routine physical work, whereas among diabetic patients, 57\% reported to have lighter type of routine physical work. Only 8\% of patients having heavy type of routine physical work were found to have diabetes mellitus. Chronic constipation and habit of smoking were found more prevalent among diabetics
as compared to nondiabetics. Further, it was observed that more than 60% of both diabetics and nondiabetics were having a routine habit of taking sleep during daytime and had higher percentage of stress. Thus, the risk factors (sedentary lifestyle and smoking), for development of diabetes, were significantly higher among diabetics as compared to nondiabetics. The frequency of night micturition among diabetics was significantly higher as compared to nondiabetics. The frequency of day micturition was higher among diabetics as compared to nondiabetics but it was not statistically significant. Feeling of pain perception in upper and lower arms, polyphagia and polydipsia were more prevalent among diabetic patients as compared to nondiabetic healthy subjects.

Blood glucose was significantly higher in all the diabetic patients in the present study and this was found associated with glucosuria to some extent. Diabetic patients showed the presence of significantly higher serum insulin, systolic blood pressure, total cholesterol, serum triglycerides and ratio of total cholesterol to serum HDL-cholesterol and ratio of serum LDL-cholesterol to serum HDL-cholesterol and serum creatinine as compared to nondiabetics while initial diastolic blood pressure as well as pulse rates of diabetics were found to be comparable with that of nondiabetics. Out of 158 diabetic patients, only 6 patients developed hypertension simultaneously or within first 6 months only, however about 36% of diabetic patients had hypertension by the time they had diabetes for a period more than 5 years. A significant positive correlation of systolic blood pressure of diabetic patients was found with the duration of diabetes ($r=0.182$, $P<0.05$) and serum triglycerides of diabetic patients ($r=0.163$, $P<0.05$). Similarly a significant positive correlation was observed between diastolic blood pressure and serum triglycerides of diabetic patients ($r=0.11$, $P<0.05$). A positive correlation of serum insulin of diabetics was found with the other parameters of diabetics i.e. systolic blood pressure ($r=0.21$, $P<0.05$), diastolic blood pressure ($r=0.22$, $P<0.05$), serum cholesterol ($r=0.19$, $P<0.05$), serum triglycerides ($r=0.32$, $P<0.05$), and ratio of total cholesterol to serum HDL-cholesterol ($r=0.18$, $P<0.05$). A significant negative correlation ($r=-0.13$, $P<0.05$) was observed between serum insulin of diabetic patients and serum HDL-cholesterol. Serum creatinine of diabetics in the
present study was found to be positively correlated with serum triglycerides levels \((r=0.12, P<0.05)\), ratio of total cholesterol to serum HDL-cholesterol \((r=0.2, P<0.05)\) and ratio of serum LDL-cholesterol to serum HDL-cholesterol \((r=0.15, P<0.05)\). Further, a significant negative correlation was observed between serum creatinine of diabetic patients and serum HDL-cholesterol \((r=-0.25, P<0.05)\).

Thus, diabetic patients had hyperglycaemia, hyperinsulinaemia, disturbed lipid profile and kidney dysfunction. Insulin resistance appears to be highly prevalent among the diabetic patients under present study. Treatment, of these diabetic patients, with *E. littorale* for a period of nine months showed significant reduction of serum glucose levels. Decrease in the blood glucose in the patients started from the first month and then continued onwards until the end of the nine months. Initial value of blood glucose of diabetic patients \((204\pm10 \text{ mg/dl})\) was reduced to \(141\pm6 \text{ mg/dl}\), at the end of nine months. Thus, it appears that *E. littorale* has an antihyperglycemic action. However, normoglycaemia was not observed in all patients. Few patients had still higher blood glucose levels even after the treatment of *E.littorale* for nine months. Untreated-normotensive-diabetics (who were not taking any antidiabetic and/or antihypertensive therapies) showed the decrease of blood glucose from the initial value of \(251.6\pm28.8 \text{ mg/dl}\) to \(144.5\pm14.8 \text{ mg/dl}\). Decrease in glucose level by *E. littorale* depends on initial glucose level (before therapy) and more decrease in blood glucose was found if there was severe hyperglycaemia before starting the therapy with *E. littorale*.

Patients taking combination of sulphonylurea+biguanides were having initial blood glucose of \(177\pm16.3 \text{ mg/dl}\) which was reduced to \(116.7\pm12 \text{ mg/dl}\) after nine months of co-administration of *E. littorale* indicating the combination of *E. littorale*+sulphonyurea+biguanides may thus be effective.

There were 48 patients taking sulphonylureas. The initial dose of sulphonylurea was \(11.5\pm0.71 \text{ mg/day}\) and corresponding blood glucose level was \(195.1\pm9.8 \text{ mg/dl}\) among these patients. After treatment with *E. littorale* for
nine months the dose of sulphonylurea was reduced to 6.5±0.72 mg/day and
the blood glucose level reduced to 139.51±7.6 mg/dl in these patients. There
were 27 patients taking biguanides. A significant decrease in the dose of
biguanides (from 887±73.2 mg/day to 497.2±96.3 mg/day) and the blood
glucose (from 172.6±11.5 mg/dl to 135.6±6.8 mg/dl) was observed when the
concurrent treatment of *E. littorale* was given along with biguanides in these
patients for nine months. Similarly, reduction in the dose of insulin injections
(from 32.5±4.1 units/day to 24.5±3.5 units/day) along with decrease in the
blood glucose levels (from 233.8±38.1 mg/dl to 171.5±12 mg/dl) were
observed when *E. littorale* was co-administered. Thus, *E. littorale* not only
reduced effectively blood glucose levels but also the dose of oral and/or
injectable antidiabetics (which the diabetic patients were taking before starting
the treatment with *E. littorale*) among diabetic patients.

Five patients taking sulphonylureas along with other medications had
controlled blood glucose before starting the treatment with *E. littorale*. The
dose of sulphonylurea before starting the treatment with *E. littorale* was
(10.5±2.5 mg/day) which was reduced to 2.5±1.7 mg/day at the end of first
month of treatment with *E. littorale*. Further, at the end of 2nd month of
treatment with *E. littorale*, all these five patients had to stop their
sulphonylureas. *E. littorale* substituted the sulphonylureas among such
diabetic patients and then they continued to have normoglycaemia and have
rest of all the other parameters comparable with nondiabetics. This further,
corroborates potential antihyperglycaemic activity of *E. littorale*.

Initial serum insulin levels of diabetics were significantly higher than
nondiabetics and the treatment of *E. littorale* consistently and significantly
decreased them during nine months of treatment with *E. littorale* in all
diabetics. Initial serum insulin was 104±10 mcU/ml which was reduced to
68±3 mcU/ml at the end of ninth month. Thus, *E. littorale* improves insulin
sensitivity among type 2 diabetics.
Many of the diabetic patients were hypertensives and had severely disturbed lipogram. Treatment with *E. littorale* for nine months in these patients produced a significant reduction in systolic blood pressure (from 139±3 mmHg to 128±2 mmHg), diastolic blood pressure (from 87±1.4 mmHg to 82±0.9 mmHg), pulse rate (from 85±1.5 beats/min to 77±1.5 beats/min), serum triglycerides (from 336±23 mg/dl to 170±7.3 mg/dl), serum cholesterol (from 239±8 mg/dl to 203±5 mg/dl), serum LDL-cholesterol (from 144±7.6 mg/dl to 128±4 mg/dl), ratio of total cholesterol to serum HDL-cholesterol (from 9.3±0.5 to 5.3±0.2), ratio of serum LDL-cholesterol to serum HDL-cholesterol (from 5.6±0.4 to 3.5±0.2) and serum creatinine (from 2.0±0.1 mg/dl to 0.9±0.4 mg/dl). Low levels of initial serum HDL-cholesterol of diabetics were significantly increased (from 28±1 mg/dl to 40±1 mg/dl) due to treatment with *E. littorale* for nine months. Thus, *E. littorale* appears to reduce insulin resistance and provides nephroprotection among diabetic patients.

Few diabetic patients withdrew from the studies due to non-medical reasons mainly non-accessibility and non-compliance. Eight patients complained of flatulence and headache, 2 patients suffered from diarrhoea and 1 patient got gastric pain. Only 9 patients were real non-responders (whose blood glucose could not be reduced by treatment with *E. littorale*). Ghanvatis prepared from the aqueous extract of *E. littorale* (representing 250 mg of the dry weight of *E. littorale* powder) given to the diabetic patients (4 ghanvatis two times a day, b.i.d.) continuously for a period of nine months was well tolerable and did not produce any severe side effects except for those mentioned above in 11 patients out of total 158 patients.

In conclusion, our data suggest that insulin resistance is highly prevalent in type 2 diabetic patients in India. *E. littorale* not only reduces the dose of antidiabetic therapy (sulphonylurea, biguanides and insulin injections) taken by these patients but also reduces blood glucose and serum insulin in these diabetic patients. *E. littorale* also decreased higher systolic blood pressure, and elevated serum triglycerides and serum cholesterol. It increased serum HDL-cholesterol in diabetic patients. It also produced
nephroprotection in diabetics as revealed from the reduction in levels of elevated serum creatinine, serum urea, urine creatinine and urine urea. E. littorale, thus can be considered as a supplementary therapy for effective treatment of various complications of type 2 diabetic patients.

Considering the significant antidiabetic activity of E. littorale as revealed from animal (in IDDM and NIDDM rats) and clinical studies (in type 2 diabetic patients), the studies to identify and isolate the chemical constituents from E. littorale were initiated. The aqueous extract of E. littorale was successively extracted with different solvents of varying polarity viz. petroleum ether (60°-80° C), toluene, chloroform, ethylacetate and finally with n-butanol. The extractive values were calculated for each of the extract. All these extracts were subjected to preliminary phytochemical analysis for the detection of alkaloids, triterpenoids, anthraquinones, flavonoids, phenols and tannins using specific reagents. Precoated TLC plates of silicagel 60F254 were spotted, in the form of a band, with 20 μl of each of the extract. For TLC evaluation, nine different solvent systems were experimented with, to find suitable solvent system/s, for different extracts to obtain good resolution of the components. Developed TLC plates were observed in UV light at 254 nm and 366 nm, derivatized by anisaldehyde-sulphuric acid reagent and by dragendorff’s reagent. The plates were scanned at 254 nm and at 366nm. Peak area, absorption spectra and \( \lambda_{max} \) of the resolved bands were recorded. Relative percentage area of each of the band was calculated.

It was found that, though the extractive value of the n-butanol extract was higher, it mainly contained triterpenoids while inspite of lower extractive values, chloroform and ethylacetate extracts were found to contain more number of different chemical groups, like flavonoids, alkaloids, phenols and tannins.

Petroleum ether, toluene, chloroform, ethylacetate, n-butanol and the remaining water extract contained 3, 7, 7, 7, 5 and 3 components respectively when scanned in UV 254. Further, it was found that the petroleum ether,
toluene, chloroform, ethylacetate, n-butanol and the remaining water extract contained 1, 6, 5, 4, 5 and 3 components respectively in UV 366. The number of bands in a chromatogram, observed under UV light were generally found to be more than the number of bands in the TLC profile of the same plate after derivatization with anisaldehyde-sulphuric acid reagent. Depending on the resolution, as observed in UV light at 254 nm, solvent system containing n-hexane:ethylacetate (80:20) was found more suitable for petroleum ether extract. Similarly the solvent systems containing toluene:chloroform:methanol (45:50:5) for toluene and chloroform extracts, acetone:chloroform:water (70:30:2) for ethylacetate extract and ethylacetate:methanol:water (77:15:8) for n-butanol extract and water extract (left after extraction with all five organic solvents) were found to be suitable showing good resolution of components. In summary, the solvent system composed of ethylacetate:methanol:water (77:15:8) was found to be the best solvent system for E. littorale on the basis of resolution obtained in UV 366, UV 254 and derivatization with anisaldehyde-sulphuric acid reagent. The absorption spectra of all the bands were compared. Total six sets of spectra were found to be overlapping with one another, indicating the presence of six same or closely related components present in the total six extracts. Toluene and chloroform extracts appear to be containing six and seven components respectively. Out of them, four appear to be closely related in both the extracts (based on the overlapping pattern of the spectra and $\lambda_{\text{max}}$ of the bands of both the extracts).

In conclusion, the results of phytopharmacological analysis including HPTLC fingerprint profile of E. littorale suggest that the solvent system composed of ethylacetate:methanol:water (77:15:8) was the best among different fractions of the aqueous extract of E. littorale. Ethylacetate fraction contained eight compounds. Toluene and chloroform fractions contained seven compounds each, out of which four compounds are identical among both the extracts. Although our studies are preliminary, further activity-guided fractionation of these extracts is expected to isolate the lead compound and active principles for the antidiabetic activity of E. littorale.