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Introduction and objectives

Diabetes mellitus has been recognized as one of the serious global health problems for which there is a need to discover new drugs. Although insulin appears to be the prime factor responsible for diabetes, 90% of diabetics seem to suffer from non-insulin dependent diabetes mellitus (NIDDM). Generally in clinical practice patients with NIDDM are treated with sulfonylureas like glibenclamide, glipizide, glyclazide etc. However an anti-diabetic agent that can maintain normoglycemia for longer duration (3-5 years) in diabetic patients remains a challenge. On long term therapy with sulfonylurea or biguanides, NIDDM patients may still require injection of insulin for adequate control of glucose level. Further, in spite of anti-diabetic therapy patients may suffer from dyslipidaemia with increase in circulating triglycerides, very low-density lipoprotein (VLDL) and hence an increased morbidity and mortality due to diabetes induced cardiovascular complications. This emphasizes the urgent need for newer and better therapeutic approaches. WHO has approved the use of traditional medicines as a part of health programme. Hundreds of products are marketed in India as “natural” agents for lowering blood sugar and decreasing long term complications. According to the WHO survey 80% of the population living in the developing countries rely almost exclusively on traditional medicine for the primary health care needs. In traditional medicine, the medicinal plants play a major role and constitute the backbone of the traditional medicine. Preliminary survey from our laboratory revealed *Enicostemma littorale* to be one of the common constituent in these formulations.

*E. littorale* Blume (Gentianaceae) is a glabrous perennial herb belonging to the family Gentianaceae. It grows throughout India up to 1.5 feet in height and more frequently near the sea. It is called Chota-kirayata or Chota chirayata in Hindi, Mamejavo in Gujarati, Nagajivha in Bengal and Vellarugu or Vallari in Tamil. *E. littorale* has been used as a folk medicine for the treatment of diabetes mellitus in Western and Southern India. *E. littorale* contains catechins, sterols, saponins, steroids, triterpenoids, alkaloids and volatile oil. Some important chemical constituents include betulin, a triterpene
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sapogenin, a secoiridoid glycoside swertiamarin, monoterpenoid alkaloids like enicoflavine and gentiocrucine.

Various Ayurvedic formulations containing *E. littorale* as one of the ingredients have been shown to produce antihyperglycemic activity in various hyperglycemic rat models. It has been reported that “Phaki” (mixture of twelve indigenous plants of which *E. littorale* is a constituent) at a dose of 50 to 200 mg/kg on i.v. administration in dogs produced a dose dependent % reduction in blood sugar between 39 to 55 %. Ethnomedical studies of North Gujarat (India) reveal the use of hot aqueous extract of *E. littorale* by the tribal inhabitants for the treatment of diabetes, fever, stomach ache, dyspepsia and malaria in interior part of Gujarat. Earlier we have reported antidiabetic activity of crude extract of *E. littorale* in type II diabetic rats. It was shown that aqueous extract produces antidiabetic effect at dose of 2 g/kg p.o. in NIDDM rats. Although the crude extract of *E. littorale* has been reported to possess anti-diabetic activity on several occasions, a detailed investigation on the effect of *E. littorale* using various fraction has not been studied. Thus, the objective of the present investigation was to perform activity guided phytopharmacological analysis of *Enicostemma littorale* with special reference to diabetes mellitus using STZ-induced IDDM and NIDDM rats.

In herbal research, it is also essential to authenticate the plant and to establish phytochemical standardization with help of reliable instruments like HPTLC. Before undertaking pharmacological work, we also carried out pharmacognostic and phytochemical standardization of *E. littorale* crude extract and its various fractions using HPTLC fingerprinting. We made an attempt to isolate and characterize the compound swertiamarin from *E. littorale*. We also studied the effect of different fractions of *E. littorale* and the compound swertiamarin on lipid profile, kidney function and liver function in STZ-induced IDDM and NIDDM rats. While studying the anti-diabetic activity of various fractions and the compound isolated from *E. littorale* (Swertiamarin) it was found that *E. littorale* also possess impressive antihyperlipidaemic and hepatoprotective activity. Thus, we extended our study to investigate the effect of aqueous extract of *E. littorale* on cholesterol fed hyperlipidemia in rats and CCL_4 induced liver injury. In the end we also made an attempt to find out the mechanism of action of various activities investigated.
Six kilogram of shade-dried herb of *E. littorale* was powdered and boiled with 24 liters of water for 8 h. The aqueous extract was concentrated under reduced pressure (yield = 635.2 g). Aqueous extract (545.0 g) was further fractionated using solvents of varying polarity viz., petroleum ether [60-80°C] (0.90 g), toluene (16.2 g), chloroform (20.2 g), ethyl acetate (29.2 g) and n-butanol (128.2 g), the extract remained after the fractionation with n-butanol was residual extract (350.3 g). The extracts were concentrated under reduced pressure and air dried to remove the solvent completely.

Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques. In the last two decades HPTLC emerged as an important tool for the qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers. In the present study all the extracts were subjected to preliminary phytochemical analysis for the detection of various class of compounds like alkaloids, triterpenoids, anthraquinones, flavanoids, phenols, tannins and coumarins using specific reagents. We also did the chemoprofiling of all the extracts using HPTLC.

TLC fingerprint profiles of aqueous extract and its fraction petroleum ether, toluene, chloroform, ethyl acetate, n-butanol and residual fractions were established using HPTLC. Suitably diluted stock solution of aqueous extract, petroleum ether, toluene, chloroform, ethyl acetate and n-butanol extract were spotted on pre-coated silica gel 60 F254 TLC plates (E. Merck) using CAMAG Linomat IV Automatic Sample Spotter and the plate were developed in the following solvent systems:

1. For aqueous extract: Ethyl acetate : methanol : water (7:7:2.0:0.5)
2. For pet ether fraction: Toluene : ethyl acetate (8:2)
3. For toluene fraction: Toluene : chloroform : methanol (6:4:1.5)
4. For chloroform fraction: Chloroform : ethyl acetate : methanol (7.7:1.5:0.3)
5. For ethyl acetate fraction: Chloroform : methanol (9.5:1)
6. For n-butanol fraction: Ethyl acetate : methanol : water (7:7:1.5:0.5) respectively.
The plates were dried at room temperature and scanned using CAMAG TLC scanner 3 at UV 254 and 366 nm and R$_F$ values, absorption spectra of the resolved bands were recorded. Further, the plates were derivatised by spraying with anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 5 min, and the R$_F$ and colours of the bands resolved were recorded.

**TLC fingerprint profiles of aqueous extract and its petroleum ether, toluene, chloroform, ethyl acetate and n-butanol fractions.**

TLC of aqueous extract showed 7 quenched bands in UV 254 nm at R$_F$ 0.06, 0.20, 0.26, 0.48, 0.62 (swertiamarin), 0.74 and 0.81. Pet ether fraction showed 8 quenched bands at R$_F$ 0.09, 0.12, 0.18, 0.22, 0.27, 0.31, 0.45, 0.71. Toluene fraction showed 8 quenched bands at R$_F$ 0.06, 0.27, 0.40, 0.45, 0.52, 0.55, 0.60, 0.70, Chloroform fraction showed 8 quenched bands at R$_F$ 0.17, 0.31, 0.39, 0.47, 0.56, 0.62, 0.79, 0.86, Ethyl acetate fraction showed 7 quenched bands at R$_F$ 0.04, 0.08, 0.15, 0.22 (swertiamarin), 0.29, 0.35, 0.45, n-butanol fraction showed 8 quenched bands at R$_F$ 0.20, 0.31, 0.51 (swertiamarin), 0.59, 0.65, 0.70, 0.84.

TLC of aqueous extract showed 5 bands in UV 366 nm at R$_F$ 0.08 (green fluorescent), 0.13 (light yellow), 0.19 (light green fluorescent), 0.27 (light blue) and 0.35 (light blue). Toluene fraction showed 7 bands in UV 366 nm at R$_F$ 0.15 (light blue), 0.36 (light blue), 0.38 (light blue), 0.45 (green fluorescent), 0.47 (light yellow), 0.54 (light green), 0.61 (light green fluorescent). Chloroform fraction showed 4 bands in UV 366 nm at R$_F$ 0.17 (green fluorescent), 0.22 (light yellow), 0.83 (blue fluorescent), 0.89 (blue fluorescent) and n-butanol fraction showed 10 bands in UV 366 nm at R$_F$ 0.10 (green fluorescent), 0.21 (light green), 0.35 (light blue), 0.46 (light blue), 0.56 (light blue), 0.61 (light green fluorescent), 0.66 (blue), 0.74 (blue), 0.79 (blue), and at 0.87 (light blue fluorescent).

TLC of aqueous extract showed 7 bands after derivatisation at R$_F$ 0.11 (orange red), 0.22 (light green), 0.36 (pink), 0.47 (light orange), 0.79 (light yellow), 0.82 (light pink), 0.88 (purple). Pet ether fraction showed 12 bands after derivatisation at R$_F$ 0.22 (light blue), 0.27 (blue), 0.30 (brown), 0.32 (blue), 0.34 (light orange red), 0.37 (blue), 0.41 (light purple), 0.44 (light orange red), 0.48 (purple), 0.55 (light purple), 0.60
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(orange), 0.67 (light orange). Toluene fraction showed 12 bands after derivatisation at Rf 0.15 (light blue), 0.18 (light blue), 0.19 (light orange), 0.25 (orange), 0.37 (yellow), 0.38 (light brown), 0.43 (light blue), 0.51 (light blue), 0.63 (light blue), 0.69 (light blue), 0.73 (light purple), 0.84 (Violet), 0.97 (Violet). Chloroform fraction showed 6 bands after derivatisation at Rf 0.09 (brown), 0.36 (violet), 0.59 (grey), 0.84 (light pink), 0.93 (pink), 0.97 (grey). Ethyl acetate showed 8 bands after derivatisation at Rf 0.24 (light green), 0.27 (pink), 0.29 (violet), 0.35 (blue), 0.37 (purple), 0.50 (blue), 0.51 (blue), and at 0.57 (light pink) and n-butanol fraction showed 8 bands after derivatisation at Rf 0.18 (light green), 0.27 (purple), 0.37 (violet), 0.49 (purple), 0.66 (light yellow), 0.75 (light yellow), 0.88 (pink), and at 0.97 (brown). None of these characteristics have been reported earlier. We repeated this profile and it was same. Hence it can be taken as one of the tool for the standardization of the plant material of *E. littorale*.

Male Sprague Dawley rats in the body weight range of 200-250 g were used. Type I diabetes was induced by injection of STZ (40 mg/kg) into the tail vein. To induce NIDDM a single dose of injection of STZ (90 mg/kg, i.p.) was given to 2 day old pups. A group of pups received only saline which served as diabetic control. After a period of 3 months, they were checked for fasting glucose levels to confirm the status of NIDDM. Animals showing glucosuria more than 2% or blood glucose level more than 140 mg/dl were selected for the experiment. Animals were divided into four groups namely non diabetic control, non diabetic treated, diabetic control and diabetic treated (n = 6-7 in each group). Diabetic treated group received aqueous extract of *E. littorale* at the dose of 0.5, 1 or 2 g/kg, p.o or toluene, chloroform, residual fraction (0.5 g/kg p.o.) or ethyl acetate, n-butanol fraction (0.1 or 0.5 g/kg p.o.) daily for three weeks. Control group received distilled water.

STZ produced cardinal signs of diabetes-mellitus such as significant loss of body weight, polyuria and polydypsia in type I diabetic rats. In STZ-diabetic rats, there was significant decrease in serum insulin levels and AUC insulin associated with significant increase in fasting blood glucose level and AUCglucose. Treatment with aqueous extract of *E. littorale* (1 g/kg or 2 g/kg) and its ethyl acetate and n-butanol fraction in the doses of (0.5 g/kg) significantly reduced the elevated food intake, water intake, glucose and AUCglucose levels of type I diabetic rats. A significant increase in serum cholesterol, serum
triglyceride, serum creatinine, serum urea, SGPT and SGOT levels observed in the type I diabetic rats. Treatment with aqueous extract of *E. littorale* (1 g/kg or 2 g/kg) or its ethyl acetate or *n*-butanol fraction in the dose of (0.5 g/kg) significantly decreased the elevated levels in type I diabetic rats. At the dose of 0.5 g/kg aqueous extract produced a significant decrease only in serum glucose, triglycerides and creatinine levels. Whereas serum cholesterol, urea and AUC$_{\text{glucose}}$ were not altered by this dose. Treatment with aqueous extract at 2 g/kg showed increased GLUT-4 levels in type I diabetic rats.

**Streptozotocin induced neonatal type 2 diabetic rats:** Fasting glucose and insulin levels in NIDDM rats were significantly (P<0.05) higher than control rats and they were significantly decreased by treatment with aqueous extract of *E. littorale* at all the doses tested and its ethyl acetate and *n*-butanol fractions. Results of oral glucose tolerance test (OGTT) showed that aqueous extract and its *n*-butanol and ethyl acetate fractions significantly (P<0.05) decrease both AUC$_{\text{glucose}}$ and AUC$_{\text{insulin}}$ values in NIDDM treated groups. Insulin sensitivity ($K_{\text{ITT}}$) index of NIDDM control was significantly lower as compared to normal control and this was significantly (P<0.05) increased after treatment with aqueous extract, its ethyl acetate and *n*-butanol fractions. Treatment with aqueous extract of *E. littorale* and its *n*-butanol and ethyl acetate fractions lowered the elevated cholesterol and triglyceride levels observed in NIDDM rats. Treatment with aqueous extract of *E. littorale* or its *n*-butanol fraction showed significant decrease in creatinine, urea, SGPT and SGOT levels as compared to NIDDM control rats. However ethyl acetate fraction showed significant changes only in creatinine and SGOT levels, and not in the levels of urea, and SGPT as compared to NIDDM control rats. Treatment with toluene, choloform and residual fractions of *E. littorale* did not produce any effect on glucose, insulin, triglyceride, cholesterol, creatinine, urea, SGPT or SGOT levels as compared to NIDDM control rats.

In preliminary TLC experiments, swertiamarin was found to be one of the major compounds in *E. littorale* it was 7.7 % in aqueous extract 5.6 % in ethyl acetate and 29.4 % in *n*-butanol fractions. TLC finger printing of the aqueous extract and its ethyl acetate and *n*-butanol fraction showed relatively higher percentage of swertiamarin. It was 43.7 % in the aqueous extract where as ethyl acetate and *n*-butanol fractions showed 10.71 % 52 % respectively. In the present work, a simple, sensitive HPTLC method was
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developed for the estimation of swertiamarin. Since swertiamarin has solubility in solvents of very varying polarity, it was a challenging task to devise a suitable solvent system to resolve it from a mixture in sample extracts. Of the several solvent systems tried, the solvent system of ethyl acetate-methanol-water (7.7 + 1.5 + 0.5, v/v) provided good separation of swertiamarin (R_F = 0.62) from the other components present in the sample extracts. The identity of the band of swertiamarin in the sample extract was confirmed by overlapping its UV absorption spectrum with that of standard swertiamarin. The purity of the swertiamarin band in the sample extracts was confirmed by comparing the absorption spectra of start middle and end position of the band.

The HPTLC method was validated in terms of precision, accuracy and repeatability. The method is specific as it resolved swertiamarin with R_F value of 0.62, despite the presence of other components in samples of _E. littorale_. A linear relationship was obtained within the concentration range of 320-1120 ng/spot for swertiamarin with the correlation coefficient of 0.99. The instrumental precision was studied by repeated scanning of the same spot seven times (% CV = 0.95). Repeatability of the method was tested by analysing the standard solution (320 ng / spot) five times (% CV = 0.09). Variability of method was studied by analysing aliquots of different concentrations on the same day (intra-day precision) and on different days (inter-day precision) and the RSD indicated that the method was precise. Accuracy of the method was determined at two levels (50 % and 100 % addition) by adding a known amount of swertiamarin to the powder of _E. littorale_ and the mixture was analysed. The recovery were found to be 101.04 % and 99.22 % at the two levels respectively and the average recovery was 100.13.

As swertiamarin was one of the major components present in more active ethyl acetate and _n_-butanol fraction. We made an attempt to isolate it. Swertiamarin was isolated by fractionation and column chromatography. This was then purified by recrystallization. Purity of isolated swertiamarin was confirmed by following methods. First was the TLC finger printing. The TLC profile showed a single spot with its R_F value varying from 0.2 and 0.9 in different solvent systems like ethyl acetate:methanol: water (9: 0.8: 0.2) showing swertiamarin at R_F 0.32, ethyl acetate:methanol: water (7.7 :1.5: 0.5)
showing swertiamarin at R_f 0.51 and ethyl acetate:methanol: water (5: 1.5: 1) showing swertiamarin at R_f 0.92.

The UV absorption spectrum of swertiamarin recorded at start, middle and end positions of the band completely overlapped, and gave an absorption maxima (λ_max) of 240 nm. Further, the TLC chromatogram also showed a single peak. The isolated sample of swertiamarin was then identified from its spectral data using UV, IR, Mass and NMR which matched well with that of the standard swertiamarin. The IR, UV and NMR spectra of the isolated sample and the standard were superimposable. The above spectral data of the isolated sample and the reference standard confirm the identity of the isolated compound as swertiamarin.

We also carried out a comparative study for the aqueous extract and the swertiamarin isolated in the type I diabetic rats. Animals were divided into three groups namely non diabetic control, diabetic control and diabetic treated (n = 6 - 7 in each group). Treatment groups received aqueous extract of *E. littorale* at the dose 2 g/kg, p.o or swertiamarin (50 mg/kg i.p.) daily for two weeks. Control group received distilled water.

Treatment with aqueous extract of *E. littorale* 2 g/kg or swertiamarin 50 mg/kg did not show significant reduction in the elevated food intake and water intake but produced significant decrease in glucose and AUC_{glucose} levels of diabetic rats. The decrease in glucose and AUC_{glucose} with aqueous extract was more as compared to swertiamarin. Treatment with aqueous extract of *E. littorale* or swertiamarin also significantly decreased STZ-induced serum cholesterol, triglycerides, LDL, VLDL and urea elevated levels in diabetic rats. The improvement in the lipid profile with swertiamarin treatment was more as compared to that of aqueous extract. Swertiamarin did not produce a significant decrease in serum creatinine, GPT and GOT levels, however, treatment with aqueous extract showed significant decrease in serum GPT and serum GOT levels. These results suggest that swertiamarin possesses anti-hyperlipemic activity. Some other constituents are likely to be present in aqueous extract of *E. littorale* that are responsible for activites not observed with swertiamarin.

In conclusion we found that antidiabetic activity observed with *E. littorale* correlates well with activity of swertiamarin. Swertiamarin is reported to have a CNS
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depressant and anticholinergic activity this makes swertiamarin as a biomarker. Further, it is an interesting molecule and a simple method for its extraction and isolation would aid in isolation of the compound in sufficient amounts for carrying out experiments to explore its biological activities since most of the Gentianaceae members are important medicinal plants. A comparative study was done for aqueous extract of *E. littorale* and the swertiamarin isolated in type I diabetic rats. Animal experiments with the aqueous extract or swertiamarin in particular showed improvement in lipid profile in STZ-diabetes. There are also no pharmacological data available to substantiate the therapeutic value of *E. littorale* in lipid disorders. In the present investigation animals fed on high cholesterol diet showed significant increase in cholesterol, triglyceride, VLDL and LDL and a decrease in HDL-C as compared to control animals. Treatment with aqueous extract of *E. littorale* or atorvastatin showed significant decrease in cholesterol, triglyceride, VLDL and LDL levels. Treatment with aqueous extract (2 g/kg p.o.) showed significant increase in HDL-C levels, however treatment with atorvastatin showed no significant increase in HDL-C levels. Animal fed on high cholesterol diet also showed increase atherogenic index as compared to control groups. Treatment with aqueous extract of *E. littorale* or atorvastatin showed significant decrease in atherogenic index. Treatment with aqueous extract at 500 mg/kg however, did not show significant decrease in atherogenic index. Treatment with aqueous extract or atorvastatin also showed antioxidant activity.

Animals fed with high cholesterol diet also showed significant increase in lipid peroxidation and superoxide dismutase levels in liver tissue when compared to control groups. Treatments with atorvastatin significantly decrease lipid peroxidation and superoxide dismutase levels; however treatment with aqueous extract of *E. littorale* showed significant decrease in lipid peroxidation levels but no change superoxide dismutase levels.

Animal fed with high cholesterol diet showed significant decrease in catalase and glutathione levels in liver as compared to control. Treatment with aqueous extract or atorvastatin showed significant increase catalase and glutathione levels in liver. *In vitro* study of aqueous extract of *E. littorale* was found to be good scavenger of DPPH radical with an EC50 of 97.70 μg/ml.
Iridoids, a widely distributed class of natural product have shown encouraging biological activities including hepatoprotective, swertiamarin a secoiridoid glycoside one of the major compounds in *E. littorale*. There are no pharmacological data available to substantiate the therapeutic value of *E. littorale* in liver disorders. In present study we also studied the effect of *E. littorale* against experimental hepatotoxicity. Hepatoprotective effect of aqueous extract was studied on carbon tetrachloride (CCl₄) induced acute liver damage in mice. Silymarin (100 mg/kg) was used as a reference standard.

Aqueous extract of *E. littorale* protected the liver from CCl₄-induced liver damage at the dose of 250, 500 and 1000 mg/kg. It also produced a significant reduction in Serum GPT, GOT and ALP activity. Besides, significant reduction in these enzymes we also found a reduction in total bilirubin and direct bilirubin levels, as compared with the control group. *E. littorale* also produced a decrease in pentobarbitone-induced sleeping time that was prolonged by CCl₄-induced liver damage. Aqueous extract of *E. littorale* produced protection of liver from CCl₄-induced diffuse necrosis, inflammatory changes, lymphocytic infiltration, vacuolation and neutrophil infiltration.

In conclusion, our data suggest that aqueous extract of *E. littorale* and its ethyl acetate and n-butanolic fractions possess potential antidiabetic activity in both the models of diabetes. Ethyl acetate and n-butanol fraction were found to be more active as compared to aqueous extract. Swertiamarin was found to be one of the major compounds in the aqueous extract and its ethyl acetate and n-butanol fractions. Swertiamarin was also found to possess potential antidiabetic activity in type I rats. The anti-diabetic activity and beneficial effect of *E. littorale* appears to be mainly due to its insulin sensitizing effects, involving increased GLUT-4 expression and antioxidant activity. *E. littorale* did not exert any toxic effects in STZ-induced impaired kidney and liver functions. It was rather found to be improving kidney and liver functions. Aqueous extract of *E. littorale* also showed anti-hyperlipidaemic in high cholesterol fed animals and also hepatoprotective effects in CCl₄-induced hepatic injury model. Active constituents of *E. littorale* responsible for hepatoprotective and anti-hyperlipidaemic activites requires further to be investigated.