

In vitro Antidiabetic Activity of Polar and Nonpolar Solvent Extracts from *Leucas aspera* (Willd.) Link Leaves

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ABSTRACT

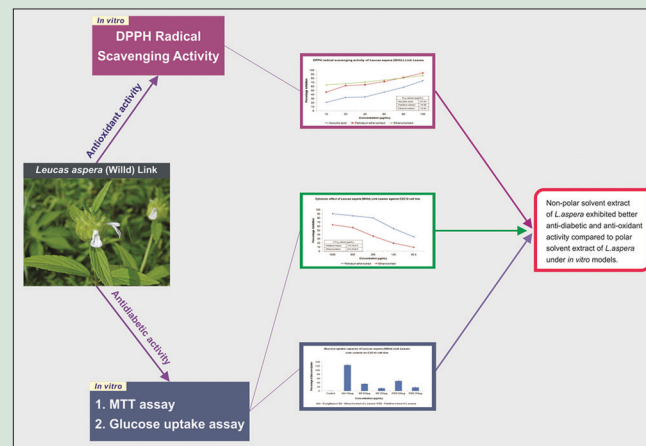
Background: Diabetes mellitus is a chronic illness, and the management of diabetes is a global problem. Successful treatment is required to prevent complications and organ damages. Herbal medicines are having minimal adverse effects when compared to the available synthetic drugs to treat such chronic diseases and disorders. **Objective:** The present study was aimed to evaluate the antidiabetic and antioxidant activity of polar and nonpolar solvent extracts of *Leucas aspera* (Willd.) link leaves under *in vitro* models. **Materials and Methods:** The *in vitro* antidiabetic activity of petroleum ether (nonpolar) and ethanol (polar) extracts were evaluated in C2C12 cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (cell viability method) and glucose uptake assay. 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method used for the evaluation of *in vitro* antioxidant activity. **Results:** Both the polar and nonpolar solvent extracts of *L. aspera* had shown better antioxidant activity compared to standard ($IC_{50} = 18.96$ and $19.90 \mu\text{g/mL}$, respectively). Petroleum ether extract exhibited better cytotoxic activity in C2C12 cell line compared to ethanol extract (concentration of test drug needed to inhibit cell growth by 50% 110.75 ± 5.5 vs. $415.25 \pm 8.0 \mu\text{g/mL}$) whereas ethanol extract showed enhanced glucose uptake activity than petroleum ether extract in C2C12 cell line at same concentrations. **Conclusion:** From our study results, we concluded that *L. aspera* (Willd.) link leaves had shown better antidiabetic activity and antioxidant activity under *in vitro* models. Nonpolar solvent extract produced slightly better activity than polar solvent extract. This study warrants further research and experiments on animal models.

Key words: *In vitro* antidiabetic, *in vitro* antioxidant, *Leucas aspera*, nonpolar solvent, polar solvent

SUMMARY

- Petroleum ether extract of *Leucas aspera* (PELA) exhibited slightly higher 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity compared to ethanol extract of *L. aspera* (EELA)
- PELA exhibited better cytotoxic activity in C2C12 cell line compared to EELA
- EELA had shown enhanced glucose uptake activity than PELA in C2C12 cell line at same concentrations

- L. aspera* leaf extracts have potential scavenging of DPPH radicals similar to that of ascorbic acid
- Overall, PELA (nonpolar) produced slightly better antidiabetic activity and antioxidant activity than EELA (polar).



Abbreviations Used: DM: Diabetes mellitus, EELA: Ethanol extract of *Leucas aspera*, *L. aspera*: *Leucas aspera*, PELA: Petroleum ether extract of *Leucas aspera*.

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INTRODUCTION

Diabetes mellitus (DM) is a group of disorders characterized by decrease in the carbohydrate metabolism, whereas lipid and protein metabolism will be increased, which leads to hyperglycemia. Virtually DM is either due to insulin deficiency or insulin resistance. Only 5% of the diabetes in the world is Type 1 (insulin dependent), and the remaining 95% falls in Type 2 (noninsulin dependent). The prevalence of diabetes is increasing globally, particularly in developing countries. Conventionally, DM was treated with herbal remedies (plants), diet, and physical exercise. Thousands of plants were used to control DM, though one-third only investigated for phytochemicals and its pharmacological activities.^[1]

Leucas aspera (Willd.) link belongs to Labiatae (Lamiaceae) family and commonly found throughout India and Philippines as well as the plains of Mauritius and Java. It has many vernacular names depending on the region and most commonly known as Thumbai.^[2] *L. aspera* is known for various pharmacological activities.^[3] The juice of *L. aspera* leaves is used as remedy for chronic skin eruptions, chronic rheumatism, and

psoriasis. In villages, leaves of *L. aspera* are used as insecticides and mosquito repellents.

In this current study, we have evaluated the *in vitro* antidiabetic activity of crude extracts obtained from *L. aspera* leaves. The main aim of this *in vitro* study was to elucidate the antidiabetic activity of a nonpolar solvent (petroleum ether) and a polar solvent (ethanol) extracts by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (cell viability method) and *in vitro* glucose uptake assay using C2C12 cell lines.

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MATERIALS AND METHODS

Plant material

Well-grown plants of *L. aspera* were collected from Kandarakottai village (Dindigul district, Tamil Nadu, India), between November 2014 and January 2015. The plant was authenticated (PARC/2014/2272) by Prof. P Jayaraman, Director, Institute of Herbal Botany and Anatomy Research Centre, Tambaram, Chennai. Leaves were then isolated, cleaned, and dried at sun shadows. Dried leaves were crushed into powder and passed through pharmaceutical sieve no 40 and 80. Any materials which passed through sieve 40 and retained at sieve 80 were collected and used for extraction.

Preparation of crude extracts

Ethanol was used as polar solvent, and petroleum ether was used as nonpolar solvent for the extraction process. Six hundred grams of the powdered leaf was soaked in 1.5 L of 70% ethanol for 72 h. After soaking, the mixture was heated at $30^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 60 min. Heated solution was cooled to room temperature and filtered through Whatman filter paper and muslin cloth. Excess solvent were then removed by distillation method. The crude extract was dried in a china dish and stored in a glass bottle at 20°C for further studies.

Six hundred grams of powdered leaves were extracted with 2.5 L of petroleum ether (60°C – 80°C) using Soxhlet apparatus (continuous extraction process). Excess solvent has been removed by distillation method, and crude extract material was dried in vacuum desiccators and stored in a glass bottle at 20°C for further studies.

Qualitative screening of phytochemicals present in *Leucas aspera* leaves

Freshly prepared crude extracts were tested for flavonoids, alkaloids, steroids, glycosides, saponins, tannins, phenolic components, fixed oils, carbohydrates, proteins, and terpenoids. The screening procedure was followed as described in textbook by Harborne.^[4]

Assessment of *in vitro* antioxidant activity of *Leucas aspera* leaves

1,1-diphenyl-2-picrylhydrazyl radical scavenging activity

The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured in terms of hydrogen-donating or radical-scavenging ability using stable DPPH radical, method described by Blois.^[5,6] One mL of 0.1 mM DPPH solution in ethanol was added to 3.0 mL of extract solution and volume made up with water at different concentrations (10–100 $\mu\text{g/mL}$). Thirty minutes later, absorbance was measured at 517 nm. Lower absorbance indicates the higher free radical scavenging activity. Capability to scavenge the DPPH radical was calculated using the following equation. Mean values were obtained from triplicate of experiment values.

$$\% \text{ inhibition} = ([\text{Control OD} - \text{Sample OD}] / \text{Control OD}) \times 100$$

Where OD: Optical density.

In vitro antidiabetic activity of *Leucas aspera* leaves extracts in C2C12 cell line

Chemicals

MTT, fetal bovine serum (FBS), phosphate-buffered saline (PBS), bovine serum albumin (BSA), D-glucose, Dulbecco's Modified Eagle's Medium (DMEM), metformin, trypsin (Sigma-Aldrich Co., St. Louis, USA), ethylenediaminetetraacetic acid (EDTA), antibiotics, insulin, dimethyl sulfoxide, NaOH, and propanol.

Cell lines and culture medium

Stock cells of C2C12 (Rat skeletal muscle, ATCC, USA) were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100 IU/mL), streptomycin (100 $\mu\text{g/mL}$), and amphotericin B (5 $\mu\text{g/mL}$) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks, and all experiments were carried out in 96 microtiter plates (Tarsons India Pvt. Ltd., Kolkata, West Bengal, India).

Test solution

For *in vitro* antidiabetic studies, test substance dissolved in DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1.0 mg/mL. This solution was sterilized by filtration and two-fold serial dilutions are prepared to carryout cytotoxic studies.

Determination of cell viability by

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

The cytotoxic capacity of ethanol extract of *L. aspera* (EELA) and petroleum ether extract of *L. aspera* (PELA) were determined by MTT assay. This method was adopted from the procedure explained by Denizot and Lang.^[7] Monolayer cell culture was trypsinized, and cell count was adjusted to 1.0×10^5 cells/mL using DMEM containing 10% of FVS. To each well, 0.1 mL of diluted cell suspension was added. Supernatant was flicked off after 24 h and monolayer was washed once with medium. Different concentrations of extracts (100 μL) were added on to the partial monolayer in microtiter plates. The plates were incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations noted at 24-h interval. After 24 h, the drug solutions in the wells were discarded and 50 μL of MTT in PBS was added to each well and gently shaken. This mixture is again incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 μL of propanol was added to the plates, gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

%Growth inhibition

$$= 100 - \left(\frac{\text{Mean optical density of test group}}{\text{Mean optical density of control group}} \right) \times 100$$

In vitro glucose uptake assay

Glucose uptake activity of EELA and PELA were measured in differentiated C2C12 cells. This method was explained by Takigawa-Imamura *et al.*^[8] and Yap *et al.*^[9] The 24-h cell culture with 70%–80% confluency in 40 mm petri dish plates was allowed to differentiate by maintaining in DMEM with 2% FBS for 4–6 days. The differentiated cells were serum starved over a night, and at the time of experiment, cells were washed with HEPES-buffered Krebs-Ringer-Phosphate solution (KRP buffer). The mixture is incubated with KRP buffer with 0.1% BSA for 30 min at 37°C . Cells were treated with different nontoxic concentrations of standard drug and extracts for 30 min along with negative controls at 37°C . D-glucose solution (20 μL) was added simultaneously to each well and incubated at 37°C for 30 min. After incubation, the uptake of the glucose was terminated by aspiration of solutions from wells and washed thrice with ice-cold KRP buffer solution. Cells were lysed with 0.1 M NaOH solution, and an aliquot of cell lysates was used to measure the

cell-associated glucose. The glucose levels in cell lysates were measured using glucose assay kit (ERBA). Two independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls.

RESULTS

Preliminary phytochemicals screening

The present study was carried out on two different crude extracts obtained from *L. aspera* leaves. The results confirmed that *L. aspera* leaves contain flavonoids, alkaloids, glycosides, carbohydrates, and less extent of steroids, terpenoids, tannins, phenolic compounds. Complete details of phytochemicals present in ethanol extract and petroleum ether extract are shown in Table 1.

In vitro antioxidant study

1,1-diphenyl-2-picrylhydrazyl scavenging activity

Both EELA and PELA exhibited a significant dose-dependent DPPH scavenging activity [Figure 1]. PELA had shown 66%, 68%, 69%, 70%, 71%, and 72% of scavenging activity at 10, 20, 40, 60, 80, and 100 µg/mL concentrations, respectively, whereas EELA had shown 64%, 67%, 71%, 76%, 81%, and 87% at same concentrations. Ascorbic acid standard exhibited 21%, 33%, 34%, 46%, 58%, and 74% of activity at the same concentrations. The percentage of scavenging activity or percentage inhibition was calculated by linear regression method. The IC₅₀ values of ascorbic acid, PELA, and EELA were found to be 27.05 µg/mL, 18.96 µg/mL, and 19.90 µg/mL, respectively.

In vitro antidiabetic studies

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

MTT cytotoxic capacity of PELA was found to be 34.6%, 54.63.44%, 80.22%, 85.27%, and 90.49% at 62.5, 125, 250, 500, and 1000 µg/mL concentrations, respectively. EELA exhibited 9.51%, 19.44%, 36.45%, 57.07%, and 63.89% at the same concentrations. PELA had shown better cytotoxic activity than EELA against C2C12 cell line culture from rat muscle. The CTC₅₀ values of PELA and EELA were found to be 110.75 ± 5.55 µg/mL and 415.25 ± 8.0 µg/mL, respectively [Table 2 and Figure 2].

In vitro glucose uptake assay

Glucose uptake activity of PELA and EELA were determined in differentiated C2C12 cells. The percentage of glucose uptake activity against rosiglitazone standard showed PELA exhibited better activity than EELA. The glucose uptake capacity of PELA was 17.2% and 48.19% at 250 µg/mL and 500 µg/mL concentrations, respectively, whereas EELA exhibited 13% and 34.29% at the same concentrations [Table 3 and Figure 3].

Table 1: Phytochemicals present in crude extracts of *Leucas aspera* leaves

Phytochemicals	Petroleum ether extract	Ethanol extract
Alkaloids	++	++
Flavonoids	++	++
Carbohydrates	++	++
Tannins	+	+
Triterpenoids	+	–
Glycosides	+	++
Steroids	+	+
Phenolic compounds	+	+
Fixed oil and fat	+	–
Proteins	–	+
Saponins	–	+

++: Strong positive; +: Positive; –: Negative/absent

DISCUSSION

Management of DM is a global problem. Successful treatment is very important for preventing or at least delaying the onset of long-term complications. Through nature, drugs are available in the form of herbal medicines with very minimal adverse effects when compared to the available synthetic drugs to treat such chronic diseases and disorders. Such herbal drugs as therapeutic agents are a nature's boon when compared to the severe adverse effects of the allopathic medical practice for diabetes. Despite the fact that the search for a complete and permanent cure for the disease is being pursued uncompromisingly by eluding physicians and researchers, many Indian Herbal Medicinal Plants have been noticed to be used successfully in managing DM and delaying or preventing its complications. We have evaluated the herb *L. aspera* for its *in vitro* antidiabetic and antioxidant potential using two different solvent extracts. The plant *L. aspera* is a well-known herbal medicinal plant used in the Indian medicines system for treating several diseases.

The nature of antidiabetic and antioxidant activity of a plant depends on the presence of phytochemicals which include flavonoids,^[10] phenolic compounds,^[11] tannin, and alkaloids.^[12] Our phytochemical studies revealed PELA and EELA contain most of these compounds which include flavonoids, alkaloids, carbohydrate, phenolic compounds, tannins, steroids, and fixed oils.

Antioxidants offer resistance to cells and prevent the diseases through scavenging the free radical molecules, inhibiting lipid peroxidations, and by many other mechanisms.^[13] A number of plant constituents have been proven for free radical scavenging or antioxidant activities; phenolic compounds are very important plant constituents. Total phenolic compounds of many plants and antioxidant activity have positive relationship due to the presence of hydroxyl group which has a scavenging ability.^[14]

The DPPH scavenging activity of the extracts evaluated against the positive control ascorbic acid and the DPPH reduction is directly proportional to the antioxidant content in the extract. Higher antioxidant contents produced higher DPPH reduction. PELA (nonpolar) exhibited the maximum DPPH scavenging with the value of 93% at a concentration of 100 µg/mL with IC₅₀ value of 18.96 µg/mL, and it showed the antioxidant activity in a concentration-dependent manner (46%–93%) in the DPPH assay. EELA (polar) extract exhibited maximum of 87% at a concentration of 100 µg/mL with IC₅₀ value of 19.90 µg/mL and showed concentration-dependent antioxidant activity (64%–87%).

L. aspera leaf extracts have potential scavenging of DPPH radicals similar to that of ascorbic acid. It is likely due to the proton-donating ability of *L. aspera* leaf extracts and stabilizes the free radicals in association with

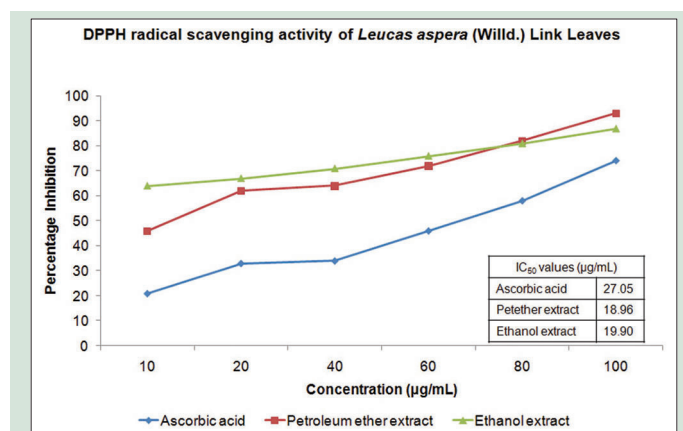


Figure 1: *In vitro* antioxidant activity of *Leucas aspera* leaf extracts

Table 2: Cytotoxic properties of *Leucas aspera* leaves against C2C12 cell line

Name of test sample	Test concentration (µg/mL)	Cytotoxicity* (%)	CTC ₅₀ (µg/mL)
Ethanol extract of <i>L. aspera</i> leaves	1000	63.89±0.9	415.25±8.0
	500	57.07±1.9	
	250	36.45±1.9	
	125	19.44±1.3	
	62.5	9.51±1.1	
Petroleum ether extract of <i>L. aspera</i> leaves	1000	90.49±1.3	110.75±5.5
	500	85.27±0.9	
	250	80.22±0.5	
	125	54.63±2.0	
	62.5	34.60±1.2	

*Values are obtained from average of 3 determinants. CTC₅₀: Concentration of test drug needed to inhibit cell growth by 50%; *L. aspera*: *Leucas aspera*

Table 3: *In vitro* glucose uptake capacity of *Leucas aspera* leaves in C2C12 cell line

Name of the test substances	Test concentration (mcg/mL)	Glucose uptake percentage (mean±SD)		
		Test 1*	Test 2*	Average
Standard (rosiglitazone)	100	124.43±6.71	125.38±2.57	124.90±4.64
Ethanol extract of <i>L. aspera</i> leaves	500	34.65±2.09	33.93±5.60	34.29±3.84
	250	12.40±3.03	13.59±3.57	12.99±3.30
Petroleum ether extract of <i>L. aspera</i> leaves	500	48.49±5.33	47.89±4.29	48.19±4.81
	250	17.75±2.00	16.65±5.27	17.2±3.63

*Test 1 and Test 2 values are obtained from average of 3 determinants. SD: Standard deviation; *L. aspera*: *Leucas aspera*

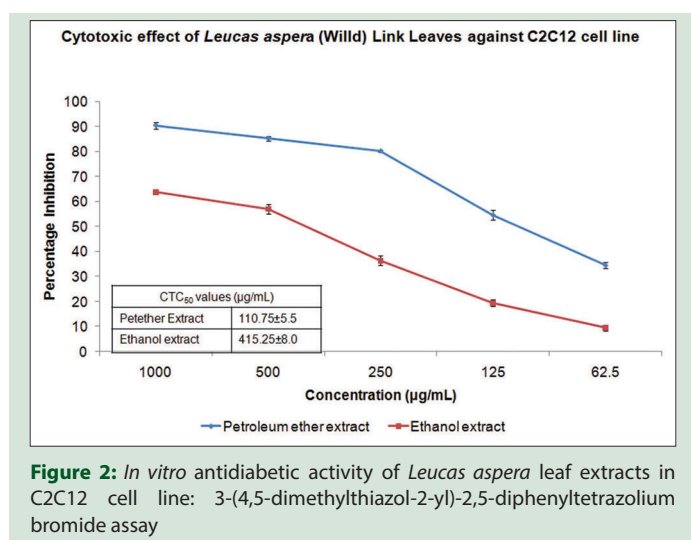


Figure 2: *In vitro* antidiabetic activity of *Leucas aspera* leaf extracts in C2C12 cell line: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

a number of hydroxyl groups.^[15] The result of this study suggests that this crude extracts of *L. aspera* leaves contain phytochemicals that are capable of donating hydrogen to a free radical.

In vitro antidiabetic studies

Skeletal muscle is the primary responsible site for postprandial glucose use, and it is the most abundant tissue in the whole body.^[16] Defects in insulin-stimulated skeletal muscle glucose uptake are common pathological states in Type 2 diabetes (noninsulin-dependent DM).^[17]

The results of the current study clearly demonstrated the antidiabetic potency of polar and nonpolar solvent extracts obtained from *L. aspera* under *in vitro* models. PELA (nonpolar) exhibited better cytotoxic activity in C2C12 cell line compared to EELA (polar). The CTC₅₀ values of PELA and EELA are 110.75 ± 5.5 and 415.25 ± 8.0 µg/mL, respectively. On the other hand, EELA had shown enhanced glucose uptake activity than PELA in C2C12 cell line at same concentrations. A study by Sah *et al.* showed petroleum ether extract of *Citrus medica* Linn. seeds-induced

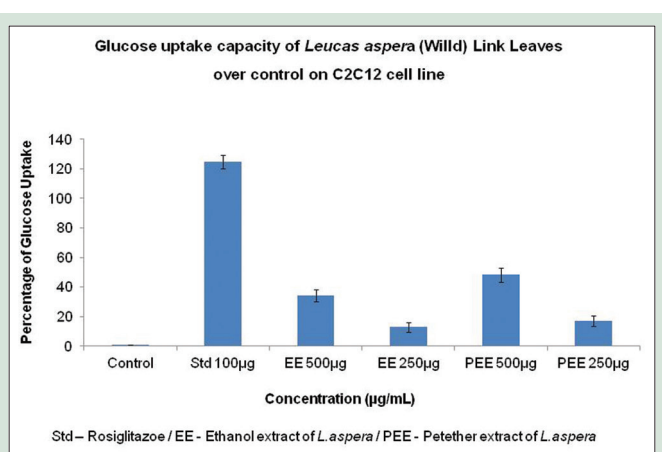


Figure 3: *In vitro* antidiabetic activity of *Leucas aspera* leaf extracts in C2C12 cell line: Glucose uptake assay

significant reduction of fasting blood glucose and lipid levels in streptozotocin-induced diabetic rats.^[18] Our study findings revealed PELA exhibited a better antidiabetic activity than ethanol extract.

CONCLUSION

From our present study results, we concluded that PELA and EELA (Willd.) link leaves had shown better antidiabetic activity and antioxidant activity under *in vitro* models. The petroleum ether (nonpolar) extract produced slightly higher activity than ethanol (polar) extract. Our study warrants further research and experiments on animal models to assess the potency and safety before the clinical use.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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