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## The antioxidant and antihypertensive potential of *Ledebouria hyderabadensis*

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**Abstract**--*Ledebouria hyderabadensis* is a new species found in the city limits of Hyderabad, Telangana, India, in 2012. Ethanolic extract of *L. hyderabadensis* bulbs was measured for its antioxidant and antihypertensive. Phytochemical investigation revealed that the plant contains glycosides, saponins, flavonoids, tannins, carbohydrates, amino acids. Acute toxicity studies performed according to OECD 423 guidelines, where a single dose of 5, 50, 300 and 1000 mg/kg of ethanolic extract of *L. hyderabadensis* bulbs administered to rats did not displayed any toxic symptoms. Therefore 1000 mg/kg is chosen as the therapeutic dose. The ethanolic extract of *L. hyderabadensis* bulbs exhibited the DPPH and NO free radical scavenging activity in dose dependent manner than ascorbic acid with IC<sub>50</sub> value 54.08 µg/ml and 64.67 µg/ml. The *L. hyderabadensis* extract exhibited statistically significant (p < 0.05) ACE enzyme inhibition IC<sub>50</sub> values (59.64 µg/ml) when compared to the standard Captopril (IC<sub>50</sub>=24.67 µg/ml). DOCA salt treatment at the 100 mg/kg, and 200 mg/kg doses, significantly reduced the Mean Systolic Blood Pressure (MSBP) (198.8&169.4) compared with the standard Nifedipine (132.6) and the negative control (122.6).

**Keywords**--*Ledebouria hyderabadensis*, acute toxicity, free radical scavenging activity, antihypertensive activity, ACE enzyme inhibition, DOCA salt treatment.

## Introduction

*Ledebouria hyderabadensis* is a new species found in the city limits of Hyderabad, Telangana, India, in 2012 (Ramana, 2012). The existence of the plant *Ledebouria hyderabadensis* is not reported elsewhere in the state or India. The genus *Ledebouria* is a deciduous plant and is characterized by its bulbs. The plants of *Ledebouria* genus have various traditional applications but not much explored of their pharmacological and phytochemical profile (Chantal, 2006). To date, basic antimicrobial screening has been performed on gram-positive bacteria such as *S. aureus* & *B. subtilis*, gram-negative bacteria *P. aeruginosa* & *K. pneumonia*, and fungal strains *C. albicans* & *A. niger* (Veenaa, 2019). Gold and silver nanoparticles synthesized from the bulb extracts were reported to have potent antimicrobial properties against the selected microorganisms strains (Raju, 2020). Homoisoflavone has been isolated from the bulbs, and anticancer potential was estimated using MTT assay using MCF-7 (breast cancer) and DU-145 (prostate cancer) (Kumar, 2014).

Stress is an initiator of chronic illness and triggers various lifestyle disorders. Heart attack and other cardiovascular diseases are the foremost reasons for adult mortality worldwide (Unger T et al., 2020). High blood pressure is the main reason for the majority of heart problems and is increasing globally (Kearney PM et al., 2015). The lack of early diagnosis for middle-aged people increases the economic burden (Steichen O et al., 2014). One-fifth of the patients with cardiovascular diseases are only under proper blood pressure management. Though various intrinsic and extrinsic factors ignite hypertension, a healthy lifestyle reduces the severity of cardiovascular problems (Chen B et al., 2011).

Antioxidants control cellular stress by scavenging the free radicals and give protection from chronic illnesses such as inflammation and other cardiovascular diseases (Lobo, 2010 & Tungmunnithum, 2018). External support from antioxidant compounds like flavonoids is needed if the internal scavenging mechanism weakens (Lourenco, 2019). Though various synthetic antioxidants are available in the market, naturally-derived antioxidants are preferred because of their fewer side effects and more biocompatibility (Bandaranayake, 2006). As the primary medicine source, plants satisfy 70-80% of global health care needs (Ekor, 2014). The popularity of herbal nutraceuticals proliferates in developing and developed countries. Herbal therapy is believed to restore health more efficiently than synthetic medicine. But herbal medicine suffers from proper pharmacological profile and dosage (Karimi, 2015). The current investigation focuses on *in vitro* antioxidant studies and *in vitro* and *in vivo* antihypertensive potential of ethanol extract of *Ledebouria hyderabadensis* bulbs in the selected models.

## Materials and Methods

### Plant material

*Ledebouria hyderabadensis* was collected from Osmania University campus, Telangana, and authenticated by Dr. M. Sankararao, Botanical survey of India,

Deccan section, Hyderabad, India. A voucher specimen (BSI/DRC/2018-19/Tech/348) was deposited in the Herbarium for future reference.

### **Reagents and chemicals**

All the chemicals and reagents were procured from Sigma Aldrich (laboratory grade).

### **Preparation of extracts**

The bulbs of *L. hyderabadensis* were collected and dried under shade. The dried bulbs were powdered and subjected to extraction with Soxhlet apparatus with ethanol as a solvent. The crude extract solution was collected by filtration, and the solvent was evaporated by rotary evaporator and percentage yield was calculated (Dokuparthi, 2014).

### **Phytochemical screening**

The preliminary phytochemical investigation for ethanolic extract of *L. hyderabadensis* bulbs was carried out by employing standard procedures (Harborne, 1973).

### **Estimation of total phenolic content**

The reducing capacity of the plant extracts can be measured by estimating the total phenolic content of the plant extract. Total phenolic content was estimated for *L. hyderabadensis* as described by Singleton et al., (1965) with minor modifications. A stock solution was prepared for gallic acid and diluted to various concentrations with 80% ethanol ranging from 25-100 µg/mL. 10 mL of Folin-Ciocalteu (10%) reagent was incubated with Na<sub>2</sub>CO<sub>3</sub> (7.5% w/v; makeup to 10 mL) for two hours at room temperature. The absorbance was measured with a UV-visible spectrophotometer (765 nm) in triplicates, and a calibration curve was plotted. This electron transferring-based assay estimated the total phenolic content and expressed it as mg of gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g) (Singleton, 1965).

### **Estimation of total flavonoid content**

Since flavonoids are the prime antioxidant components of plants origin and may involve in the direct neutralization of the free radicals. The total flavonoid content in the ethanol extract of *L. hyderabadensis* was estimated using the AlCl<sub>3</sub> method. In this, 1mL of the standard Quercetin was incubated with one-fifth of its volume of AlCl<sub>3</sub> solution (10% w/v) and 1M potassium acetate for 30 minutes. The final volume was adjusted to 6mL with distilled water, and the absorbance was measured at 415nm wavelength using a UV-Visible spectrophotometer. The total flavonoid content was expressed as µg of quercetin equivalents per mg dry ethanol extract of *L. hyderabadensis* (µg QE/ mg dry weight) (Chang, 2020).

### **Estimation of total tannin content**

The total condensed tannins present in the ethanol extract of *L. hyderabadensis* bulbs were estimated using Vanillin and HCl reagents. To the 0.4mL of the ethanol extract, 3mL of 4% Vanillin solution prepared in ethanol was added. 1.5mL conc. HCl was added slowly to this mixture to generate red colour with the condensed tannins such as leucoanthocyanins and catechins present in the extract. The intensity of the color was measured spectrophotometrically at 500nm wavelength using UV-Visible spectrophotometer in triplicates. The total condensed tannin content was represented as mg of tannic acid equivalents per mg dry matter (mg TAE/gm) dry weight (Broadhurst,1978).

### ***In vitro* antioxidant assay**

#### **DPPH radical scavenging assay**

The ethanol extract of the *L. hyderabadensis* bulbs was screened for its ability to neutralize the *insitu* generated DPPH free radicals. 2mL of DPPH solution (0.5mM) was added with 0.2mL of the extract solution and incubated for 20 minutes at room temperature. The absorbance was measured at 515 nm for individual extracts and Ascorbic acid in triplicates. The antioxidant activity was calculated using the formula given below (BLOIS, 1958).

$$\% \text{ Free radical scavenging activity} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

#### **Nitric oxide radical scavenging assay**

Nitric oxide radical scavenging activity for the ethanolic extract was measured using the Griess reagent. To sodium nitroprusside (0.5ml) buffered solution, a test solution of various concentrations (1 ml) was added and incubated at 25°C for 3 hours. This mixture was added to the equal volumes of Griess reagent, and 150µl of this solution was transferred to the microplates. Absorbance was measured at 540nm using a UV-Visible spectrophotometer, and values are expressed as average values triplicates (Rajasekaran, 2016 & Dokuparthi,2021). Percentage of nitric oxide radical scavenging assay =  $[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{Test}}) / \text{Abs}_{\text{control}}] \times 100$

### **Acute toxicity studies**

Acute toxicity studies of ethanolic extract of *L. hyderabadensis* were evaluated following OECD guidelines-423. The animals were treated orally with the ethanol extract with a 2,000 mg/kg bodyweight limit dose. Abnormal behavior and mortality were observed.

### **Angiotensin I converting enzyme (ACE) inhibition assay**

ACE is a crucial enzyme regulating blood pressure and fluid balance by converting biologically inactive Angiotensin-I into Angiotensin-II. Since, Angiotensin-II is a potent vasoconstrictor, limiting this step can lower blood pressure and be a treatment option for people with hypertension (Chaudhary,

2015 ). The assay protocol for this experiment was adopted from the methods described by Jimsheena *et al.* with slight modifications (Jimsheena, 2011). The test solution (50  $\mu$ L) from the plant fractions was prepared using phosphate buffer (200  $\mu$ L, maintaining a pH of 8.3), sodium chloride (0.2 M), and hippuryl-histidyl-leucine (HHL, 6.5mM). This mixture was incubated at 37°C for 30 min with ACE solutions (100  $\mu$ L, 0.1 U mL<sup>-1</sup>), and the progress of the reaction was suspended by the addition of 1 M HCl (50  $\mu$ L). The hippuric acid generated from the reaction mixture was separated using ethyl acetate with subsequent centrifugation and vacuum evaporation of the solvent. The absorbance was determined for the final product at 228 nm using a spectrophotometer, and the ACE inhibition activity was calculated as

$$\text{Inhibition \%} = (A_a - A_b) \times 100 / (A_a - A_c)$$

Where  $A_a$  is the absorbance with ACE and HHL without the sample (positive control, no inhibition, and maximum activity);  $A_b$  is the absorbance with ACE, HHL, and the sample or standard; and  $A_c$  is the absorbance with HHL without ACE and the sample (control).

### **DOCA-salt-induced hypertension model**

Nephrectomy was performed on rats by making a small incision, and the left kidney was removed, followed by the ligating left renal artery, vein, and ureter. The incision was sutured, and proper post-operative care was followed for seven days. The animals also received 1% sodium chloride solution daily through drinking water and subcutaneous injection of Deoxycorticosterone acetate (DOCA)-salt (20 mg/Kg body weight) twice weekly for five weeks (Bankar, 2011).

### **Measurement of blood pressure**

The tail-cuff method was used for measuring systolic blood pressure weekly to determine the effect of the treatment on the groups (Imenshahidi, 2013). The animals received prior training, and the blood pressure was measured in triplicates using a pressure transducer (Letica5002 Storage Pressure Meter). After five weeks of treatment, animals were anesthetized with sodium thiopental (45mg/kg body weight, i.p.). The blood pressure was measured from the left common carotid artery after tracheostomy.

### **Statistical analysis**

GraphPad Prism TM software was used for the analysis and the values are represented as mean  $\pm$  SEM. ANOVA (one-way) followed by Tukey-Kramer post-hoc test was used for multiple comparisons. The level of significance (p value) was considered as less than 0.05.

## Results

### Preliminary phytochemical screening

The preliminary phytochemical study of ethanolic extract of *L. hyderabadensis* revealed that the extracts are instituted with various secondary metabolites such as Alkaloids, saponins, flavonoids, tannins, amino acids, and carbohydrates (Table 1).

### Total phenolic content

Phenols can be directly interrelated to their protecting effect against the cellular stress in the body. The total phenolic content of ethanolic extract of *L. hyderabadensis* leaves was evaluated by Folin–Ciocalteu method taking gallic acid as the standard. A calibration curve was plotted with the absorbance values against different concentrations of gallic acid. Total phenolic content of the extracts was calculated from the regression equation of the calibration curve ( $6.696x + 0.2432$ ;  $R^2 = 0.9969$ ) and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g) (Table 2). Where  $y$  is the absorbance at 760 nm and  $x$  is the total phenolic content in the extracts. Total phenolic content values were observed in ethanolic extract of *L. hyderabadensis* is  $92 \pm 1.23 \text{ mgGE/g}$ .

### Total flavonoid content

Polyphenolic compounds such as flavonoids are important adaptogenic compounds that help the body to adapt to the innumerable harsh environments. These defensive agents also improve human health from chronic ailments. The flavonoid content of ethanolic extract of *L. hyderabadensis* was determined by a colorimetric method using the Dowd technique and is found to be  $46 \pm 0.81 \text{ mg}$  of gram equivalence of Quercetin at 415 nm (Table 2). The calibration curve was made by linear regression and the results were represented in triplicates. The total phenolic content of the extract was calculated from the regression equation of the calibration curve ( $Y = 7.659x + 0.0801$ ,  $R^2 = 0.9951$ ) and found as  $46 \pm 0.81 \text{ mg}$  Quercetin equivalents per gram of sample in dry weight (mg QE/g).

### Total tannin content

The total condensed tannin content was determined for ethanolic extract of *L. hyderabadensis* using the Broadhurst method and it is found that the extract is having  $187.3 \pm 1.24 \text{ mg}$  of gram equivalence of Tannic acid at 500 nm (Table 2). A calibration curve ( $Y = 7.0039x + 0.1002$ ,  $R^2 = 0.9962$ ) was plotted with various concentrations of tannic acid and found as  $12.5 \pm 1.93 \text{ mg}$  tannic acid equivalents per gram of sample in dry weight (mg TAE/g).

## **In vitro antioxidant assay**

### **DPPH radical scavenging assay**

In the present study, *L. hyderabadensis* has exhibited significant free radical scavenging activity in a dose-dependent manner when compared to ascorbic acid. A standard curve ( $Y = 6.7556x + 0.2276$ ,  $R^2 = 0.9953$ ) was plotted using various concentrations of ascorbic acid and at a higher concentration (75  $\mu\text{g/mL}$ ), the extract displayed 62.34% of inhibition ( $\text{IC}_{50} = 54.08$ ) next to ascorbic acid (80.18%;  $\text{IC}_{50} = 37.8$ ) and inferred to be a potent antioxidant (Table 3 and Figure 1). The ability of the extract to scavenge DPPH could also signify its ability to confront stress and subsequently the protective effects in the body.

### **Nitric oxide radical scavenging activity**

When compared to the standard Ascorbic acid, *L. hyderabadensis* ethanolic extract exhibited significant NO free radical scavenging activity in a dose-dependent manner (Table 3 and Figure 2). At higher concentration (75  $\mu\text{g/mL}$ ), the extract is showing 54.27% of inhibition with  $\text{IC}_{50}$  values 64.67  $\mu\text{g/mL}$ . Whereas for ascorbic acid, it is 77.16% with  $\text{IC}_{50}$  values 44.32  $\mu\text{g/mL}$ . The polyphenolic compounds such as tannins and flavonoids present in the *L. hyderabadensis* may neutralize the free radicals liberated by the nitroprusside in the given procedure and may offer protection in the body linked to cellular stress.

### **Acute toxicity studies**

Acute toxicity was investigated under OECD guidelines 423. The extract was administered to female rats at doses 5, 50, 300 & 1000 mg/kg with oral gavage did not display any symptoms of toxicity. The rats were examined for two weeks, twice a day has not exhibited toxic signs. Hence oral  $\text{LD}_{50}$  was finalized to surpass 1000 mg/kg. Therefore 1000 mg/kg was regarded as the safest higher dose for ethanolic extract, and  $1/10^{\text{th}}$ , i.e., 100 mg/kg (lower dose) and  $1/5^{\text{th}}$  of 1000 mg/kg, i.e., 200 mg/kg (higher dose), was preferred for further studies. But it is start showing toxicity at 1500mg/kg bw onwards. So, it is moderately safe to the animals

### **Angiotensin-I converting enzyme (ACE) inhibition assay**

ACE is a prime target for controlling hypertension, followed by  $\text{Ca}^{+2}$  channel blockers. The percentage inhibition of the ACE enzyme by the fractions was depicted in the Table 4. The results show that *L. hyderabadensis* exhibited statistically significant ( $p < 0.05$ ) inhibition  $\text{IC}_{50}$  values (59.64  $\mu\text{g/mL}$ ) when compared to the standard Captopril ( $\text{IC}_{50} = 24.67 \mu\text{g/mL}$ ).

### **DOCA-salt-induced hypertension model**

DOCA salt treatment significantly increased the Mean Systolic Blood Pressure (MSBP) of the experimental animals, and Nifedipine reverted the blood pressure to normal. The effect of the various fractions of *L. hyderabadensis* was studied at

100 mg/kg and 200 mg/kg of body weight for five weeks by measuring the MSBP of the unilaterally nephrectomised rats and compared with the standard (132.6) and the negative control (122.6). At the selected doses, the ethanol extract significantly decreased (198.8&169.4) the MSBP in a dose-dependent manner (Table 5).

### **Discussions & Conclusion**

Dietary polyphenolic compounds such as flavonoids, tannins may contribute to the plant detoxification system. They are used to have protective effect against inflammations, DNA damage and cardiovascular diseases by alleviating vascular health. (Pandey, 2009). Flavonoids helps to eliminate harsh chemicals by transforming into metabolic end products. Plant flavonoids have similar scavenging properties like Vitamin-C or E to control the cellular stress. Citrus fruits and Terminalia fruits are reported to have more polyphenolic compounds with known protective effects(Habauzit, 2012).

In the current research, the ethanolic extracts of *L. hyderabadensis* was evaluated for its phytochemicals using standard protocols and revealed to have significant protective agents such phenols and tannins. The antioxidant activity screening results revealed the free radical scavenging property of the extract against DPPH & Nitric oxide free radicals. The extract was found to be moderately safe to the animals and DOCA salt along with 1% NaCl treatment decreased the renal output and created high blood pressure in the animals. Animals treated with two doses of *L. hyderabadensis* extracts were effectively restored the MSBP when compared to Captopril. After six weeks of treatment, animals recovered from the hypertension indicating the antihypertensive properties of the bulbs.

From the results, it can be concluded that *L. hyderabadensis* exhibited significant antihypertensive property both *invitro* and *invivo*. The polyphenolic compounds such as flavonoids and tannins along with other secondary metabolites, may impact the reported pharmacological activity. Further investigation is needed to assess the detailed phytochemical and pharmacological profile to justify its reported antioxidant and antihypertensive property.

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Table 1. Phytochemical screening of ethanolic extract of *L. hyderabadensis*

Phytochemicals	Present (+)/absent (-)
Alkaloids	-
Glycosides	+
Saponins	+
Flavonoids	+
Steroids	-

Tannins	+
Proteins	-
Carbohydrates	+
Amino acids	+
Lipids	-

Table 2. Phytochemical profile of ethanolic extract of *L. hyderabadensis*

Type	Values
Total phenolic content	92±1.23mgGE/g
Total flavonoid content	46±0.81mgQE/g
Total tannin content	12.5±1.93 mgTAE/g

\*All values are expressed as mean±SD for three determinations

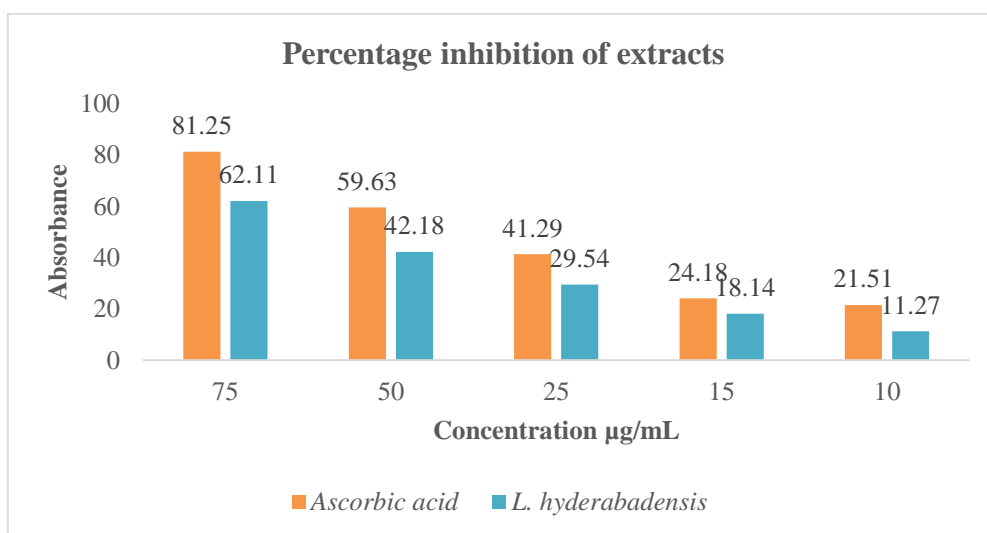


Figure 1. Percentage inhibition of extracts at different concentrations for DPPH assay

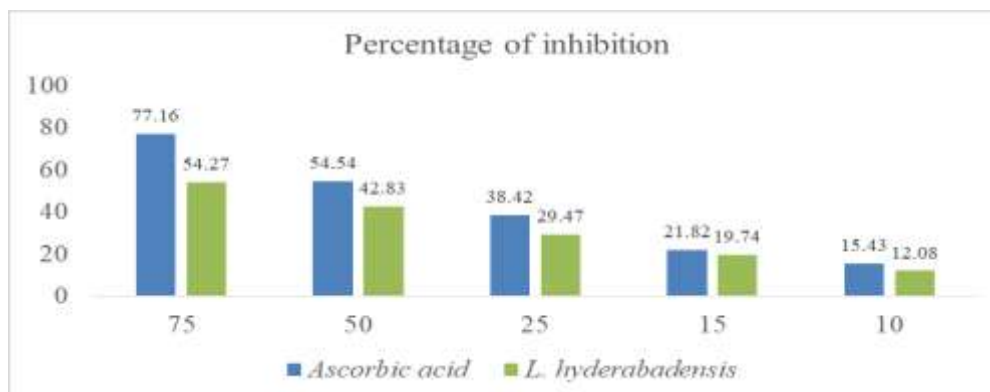


Figure 2. Percentage inhibition of extracts at different concentrations for NO free radical scavenging assay

Table 3. Percentage inhibition and IC<sub>50</sub> values of extracts and ascorbic acid at different concentrations

Concentration µg/mL	DPPH assay		NO free radical assay	
	Ascorbic acid	<i>L. hyderabadensis</i>	Ascorbic acid	<i>L. hyderabadensis</i>
75	81.25	62.11	77.16	54.27
50	59.63	42.18	54.54	42.83
25	41.29	29.54	38.42	29.47
15	24.18	18.14	21.82	19.74
10	21.51	11.27	15.43	12.08
IC <sub>50</sub> µg/ml	39.82	58.51	44.32	64.67

Table 4. Percentage inhibition of the ACE enzyme by *L. hyderabadensis*

Concentration (µg/ml)	<i>L. hyderabadensis</i>	Captopril
100	63.2±1.3	80.4±1.82
50	54.2±1.3	64.2±1.92
25	43±2.12	53.2±1.92
12.5	28.8±1.08	44.6±3.04
6.25	10.8±1.64	38±2.23
IC <sub>50</sub>	59.64	24.67

Table 5. Effect of *L. hyderabadensis* on MSBP of the unilaterally nephrectomised rats

Treatment	MSBP
Negative control	122.6±1.34
Disease control	210.4±1.14
Positive control	132.6±1.67
LHE 100µg/ml	198.8±2.58
LHE 200µg/ml	169.4±2.07