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**Surabhi Bajpai**  
Department of Bioscience and  
Biotechnology, Banasthali  
Vidyapith, Banasthali,  
Rajasthan, India

**Himani Hooda**  
Department of Bioscience and  
Biotechnology, Banasthali  
Vidyapith, Banasthali,  
Rajasthan, India

**RK Singh**  
Department of Biochemistry,  
Institute of Science, Banaras  
Hindu University, Varanasi,  
Uttar Pradesh, India

**Rakesh Mishra**  
Department of Zoology, Banaras  
Hindu University, Varanasi,  
Uttar Pradesh, India

## Comparative analysis of antioxidant properties of extracts of *Calotropis procera* with different anti-diabetic drugs

**Surabhi Bajpai, Himani Hooda, RK Singh and Rakesh Mishra**

### Abstract

Medicinal plant extracts have been implicated for the treatment of diabetes mellitus since ages. The present study deals with extraction and evaluation of various phytochemical constituents in leaf, root and stem of *Calotropis procera* and comparison of their anti-oxidant properties with antidiabetic drugs. Pet ether, hydroethanolic and aqueous extracts of *Calotropis procera* were prepared, phytochemical screening and high performance liquid chromatography profiling were done. The antioxidant properties of extracts were evaluated and compared with anti-diabetic drugs (insulin, metformin and pioglitazone). Level of phenols was significantly higher in pet ether and hydroethanolic extracts of leaves of the plant ( $p < 0.001$ ). Pet ether extracts of leaves had significantly increased DPPH (1, 1-diphenyl-2-picrylhydrazyl) scavenging and superoxide radical scavenging activity respectively ( $p < 0.001$ ). Concluding the above findings we state that *Calotropis procera* leaf has optimum antioxidant potential henceforth this plant can be used in future as a valuable source of pharmacologically active antioxidants for the control of diabetes.

**Keywords:** Free radical, anti-oxidant, *C. procera*

### 1. Introduction

Diabetes mellitus is a major global burden with 382 million people suffering from the disease by the end of 2013 and this figure is expected to raise up to 592 million by year 2035 [1]. The factors that cause diabetes include an unbalanced ratio of the reactive oxygen species and antioxidant enzymes concerned with their removal [2]. Reactive oxygen species are highly reactive ions or small molecules including oxygen ions ( $O_2$ ), free radicals ( $\cdot OH$ ) and peroxides ( $H_2O_2$ ) formed as natural byproducts of cellular energy metabolism [3]. There are evidences that demonstrate the increased production of ROS in diabetes. It is because of the reactive chemical nature of ROS due to which it oxidizes and damages DNA, protein, lipids and carbohydrates and thus believed to play a key role in pathogenesis of diabetes [4]. Further, ROS mediated hyperglycemia-induced activation of signal transduction cascades and transcription factors leads to transcriptional activation of profibrotic genes [5]. Protein kinase C (PKC), transforming growth factor- $\beta 1$  (TGF- $\beta 1$ ) and angiotensin II (Ang II) are the common factors stimulated by hyperglycemia-induced ROS, in turn, generate and signal through ROS. Therefore ROS act as a signal amplifier in diabetes [6]. It has been reported in diabetes that there is reduced plasma/serum antioxidant status or free radical scavenging activity and increased plasma oxidisability [7]. It has also been reported that total antioxidant capacity of plasma of type I diabetes patient is 16% lower than that of normal subjects.

Ethno botanical studies of traditional herbal remedies are frequently conducted for treatment of diabetes around the world. It has been identified that more than 1,200 species of plants contains hypoglycemic properties [8]. To date, over 400 traditional plants have been reported for treatment of diabetes, although only a small number of these have received scientific and medical authentication to assess their efficacy [9]. Active hypoglycemic agents have been isolated from plants like *Allium sativum*, *Gymnema sylvestre*, *Citrullus colocynthis*, *Trigonella foenum greacum*, *Momordica charantia* and *Ficus bengalensis* etc. and their mechanism of action have been studied widely [10].

*Calotropis procera* has been implicated for the treatment of a wide range of common ailments like fevers, rheumatism, indigestion, cough, cold, eczema, asthma, elephantiasis, nausea, vomiting and diarrhea [11]. *C. procera* belongs to the family Asclepiadaceae. It is a drought-resistant, salt-tolerant weed found in the arid regions of India particularly states like Rajasthan and Gujarat [12]. It is native to tropical Africa including Nigeria, Asia and Latin America. It is commonly referred to as swallow wart, milk weed, sodom apple and rooster tree.

**Correspondence**  
**Surabhi Bajpai**  
Department of Bioscience and  
Biotechnology, Banasthali  
Vidyapith, Banasthali,  
Rajasthan, India

Yoruba tribe (in Nigeria) calls it 'bomubomu' [13]. In ancient time in Ayurveda the plant *Calotropis procera* was mentioned as "Rakta-arka". This plant has been used for treatment of various diseases since the time immemorial but its role in diabetes is yet unexplored.

The following investigation is a comparative study that explores the antioxidant activity of different parts (leaf, stem and root) of *Calotropis procera* extracted in different solvents like pet ether, ethanol and aqueous extract. Partial identification of active compounds in the crude extract of *Calotropis procera* was also performed using HPLC.

## 2. Methods

### 2.1 Collection of plant material and extraction

Root, leaf and stem of *C. procera* were collected from Banasthali Vidyapith, Rajasthan, India. The plant was authenticated at Department of Biotechnology and Bioscience, Banasthali University. Collected plant samples were separated, washed thoroughly with water and shades dried 50 gm of fresh /air dried (25 °C for 5 days in absence of sunlight) and converted into fine powder using a suitable mechanical grinder.

### 2.2. Extract preparation

The dried powder 450g of root, leaves and stem was extracted in with pet ether, ethanol (75%) and water sequentially for 3 days (shaking it timely) by decoction method. The extract was then filtered by whatman filter paper (Bibby RE 200, Sterilin Ltd. UK). The filtrate was concentrated using rotary evaporator 50 °C under reduced pressure to get the solid mass [14]. The concentrated crude was lyophilized into powder (5gm). The crude extract was then suspended with three different solvents.

### 2.3 Qualitative phytochemical analysis of the extracts

Identification of some bioactive components of the extracts was done by qualitative phytochemical tests using standard methods. The main bioactive components like flavonoid (Shinoda test, Aluminium chloride colorimetric method), phenols and tannins, (Gelatin test), phlobatanins, saponins (Frothing test), terpenoids, (Keller-killani test), carotenoids were tested [15].

### 2.4 Qualitative and quantitative analysis of HPLC

The samples were analyzed by HPLC (Shimadzu, LC10A, Japan) using standard protocol. A maximum pressure of 400 kgf/cm<sup>2</sup> was maintained along with A: B solvent phase concentration of 30:70 at a wavelength of 260 nm. Run time was 15 minute. Chromatography was performed on C18 column using solvent A (2% glacial acetic acid in water) and solvent B (30% acetonitrile and 2% glacial acetic acid in water) [16].

Standards were run in concentration of 0.5 mg/ml. The phenolic acids derivatives (260nm) identified were further quantified by using standards like gallic acid and cinnamic acid. Amount of identified compounds was determined by comparison with the integration areas of the peaks of standards.

## 2.5 Determination of antioxidant properties

### 2.5.1 DPPH free radical scavenging assay

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was carried out for the evaluation of the antioxidant activity. The ability of plant extracts (leaf, root, stem) to scavenge DPPH radical was estimated by taking solution containing diluted plant extracts (1ml) and antidiabetic drugs (equal concentration) with DPPH (0.1 mM/L) methanol (1.0 ml) solution and then incubated in dark at ambient temperature for 10 min and allowed to react the mixture. After 10 min. the absorbance was recorded at 517 nm against blank the values were then change in to percentage to evaluate the antioxidant activity 1.0 ml of methanol was used as control in place of extract. Ascorbic acid was used as positive control [17].

% of radical scavenging activity = [(OD control-OD sample)/OD control] × 100

Where OD is optical density.

### 2.5.2 Superoxide anion (O<sub>2</sub><sup>-</sup>) radical scavenging activity

The super oxide radical scavenging activity was by measured by the decrease in absorbance of reaction mixture containing NBT (Nitro blue tetrazolium). The reaction mixture (2ml) was prepared by adding 1ml NBT solution (144µM in 100mM phosphate buffer, pH 7.4) 0.25ml, 1ml NADH solution (677 µM in 100 mM phosphate buffer, pH 7.4). Further, 0.25ml and 0.5ml (0-20µg/ml) of sample extracts /anti-diabetic drugs solution were mixed and then the reaction was started by adding 100µl of PMS (polymethylhydro-dimethylsiloxane) solution (60µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 minutes and absorbance was taken at 562nm against an appropriate blank to detect the formazon formation. Decreased absorbance of reaction mixture indicated increased superoxide scavenging activity. Inhibition was measured using following formula [18]. Ascorbic acid was used as positive control.

% Inhibition = [(A<sub>0</sub>-A<sub>1</sub>) /A<sub>0</sub>] × 100

Where A<sub>0</sub> was the absorbance of the control, and A<sub>1</sub> was the absorbance of the extracts and standards.

## 2.6 Statistical analysis

Statistical analyses of the experiments were performed by student's t- test followed by two way ANOVA. The graphical description was done by Microsoft excel 2007(Roselle, IL, USA). The significant level of the results was determined as *p*<0.001.

## 3. Results

### 3.1 Qualitative phytochemical analysis

The phytochemical analysis of extracts is shown in table 1. The plant extracts contained considerable amount of flavonoids, phenols, tannins, phlobatanins, carotenoids, and saponins. Significant amount of phytochemicals were estimated in root, stem and leaf (Aqueous/hydro-ethanolic/pet ether) extracts. Flavonoids were present in hydro-ethanolic and aqueous extracts of leaves and roots. Phenols were present in pet-ether extracts and hydroethanolic extracts of leaves.

**Table 1:** Qualitative phytochemical analysis profile of *C. procera* (root, stem, leaf) extracts

	Pet ether Extract			Hydroethanolic Extract			Aqueous Extract		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
Flavonoids	++	++	++	++	++	++	++	++	++
Phenol	++	++	+++	++	++	+++	++	++	++
Carotenoids	++	--	++	++	--	++	++	++	++
Phylobatannins	--	++	--	++	--	++	--	--	++
Saponins	++	++	++	++	--	++	--	++	++
Tannins	++	++	++	++	++	++	++	++	++

The sign (.) indicate the absence of compound, (+) indicate low levels of the compound, (++) indicate moderate levels of the compound, (+++) indicate significant levels of the compound.

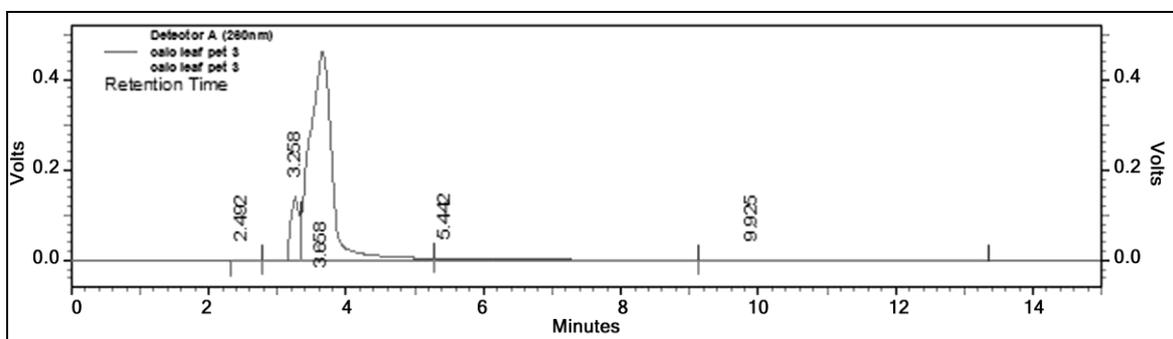
### 3.2 High Performance Liquid Chromatography

In HPLC analysis of leaf extract of *C. procera* was performed (since the phenolic activity was found significantly high in leaf extract) using standard of phenols like gallic acid and cinnamic acid. HPLC chromatograms of different extracts were compared with standards. HPLC was run for 14 minutes and absorbance was taken at 260nm. The HPLC chromatogram of leaves also contained certain other peaks with retention time different from that of standards thus

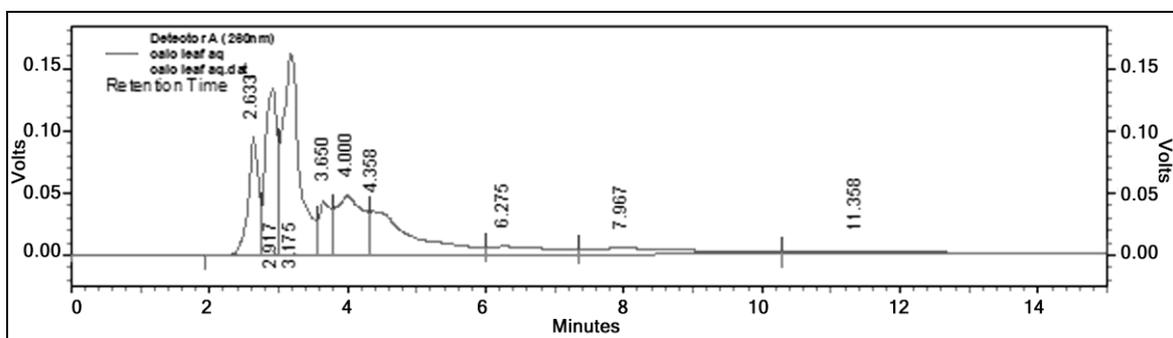
indicating the presence of other phytoconstituents as well (Fig. 1).

### 3.3 HPLC Chromatogram

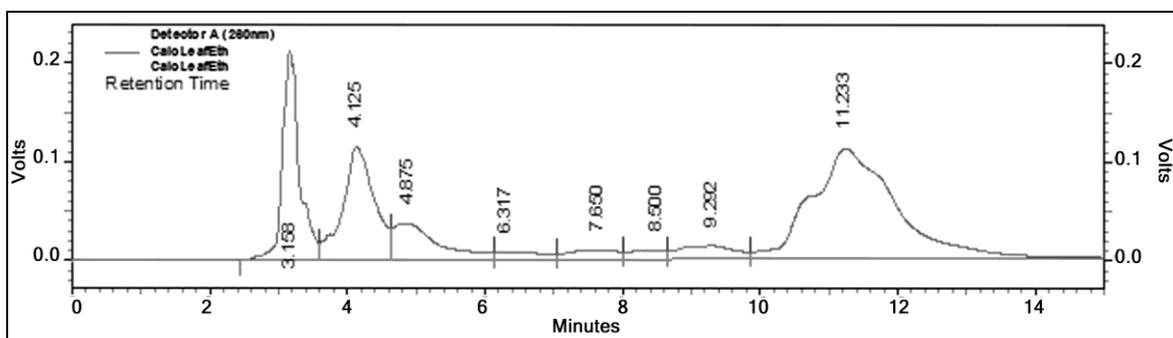
The following result shows HPLC chromatograms of pet ether, hydroethanolic and aqueous extracts of leaves of *C. procera*. Standards like gallic acid at concentration 1 (mg/ml) exhibited retention time 3.625 min. whereas cinnamic acid at 1mg/ml showed retention time of 3.422 min.



(A)



(B)



(C)

**Fig 1:** HPLC analysis of *C. procera* (A) PEE of leaves (B) HEE of leaves (C) AQE of leaves recorded at 260 nm. Solvents used were solvent A (2% glacial acetic acid in water) and solvent B (30% acetonitrile and 2% glacial acetic acid in water), with phase concentration 30:70 at a flow rate 1ml/min.

### 3.4 DPPH free radical scavenging activity

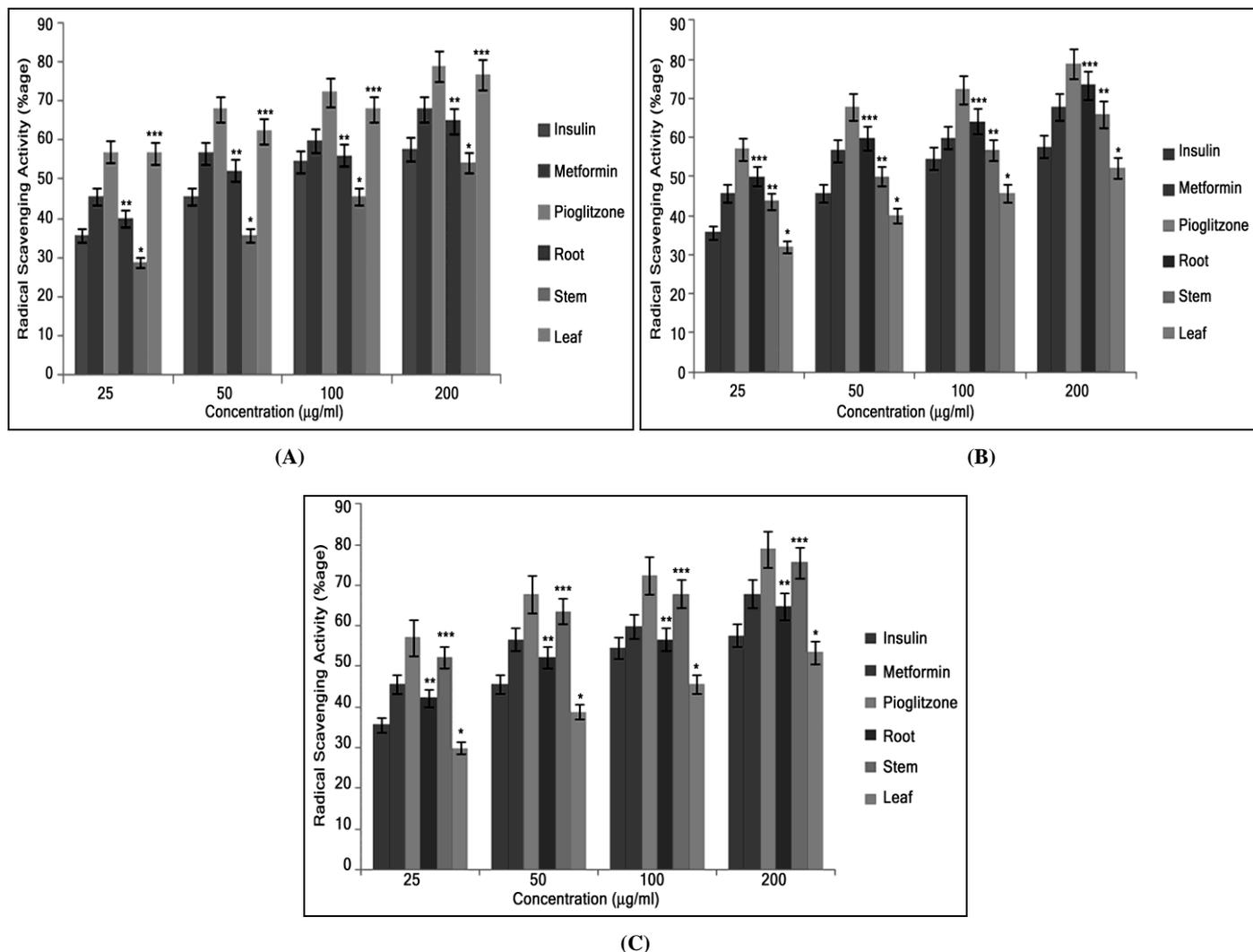
DPPH scavenging ability of leaf, stem and roots extracts of *C. procera* in comparison with different anti-diabetic drugs is represented in the figure 2. Concentration dependent radical scavenging activity was reported in leaf extract in comparison to anti-diabetic drugs. The DPPH scavenging activity order from extracts of *C. procera* was found to be in the order: PEE of leaves > HEE/AQE.

Pet-ether extracts of leaves had significantly higher scavenging capacity at concentrations 25,50, 100, 200 µg/ml, as compared to root and stem respectively ( $p<0.001$ ). At the 100µg/ml concentration the radical scavenging activity of leaf

extract groups of pet ether reached 80% approximately and the level of significance was almost equivalent to pioglitazone, the anti-diabetic drug (Fig. 2A).

Hydroethanolic extracts of leaves and roots at concentrations 25, 50, 100, 200 µg/ml did not show significant change in scavenging activity in comparison with anti-diabetic drugs (Fig. 2B).

While in aqueous extracts, concentration dependent scavenging activity was reported in stem extract as compared to root and leaf extract ( $p<0.001$ ) respectively (figure 2C). Ascorbic acid of equivalent concentration was used as reference control.

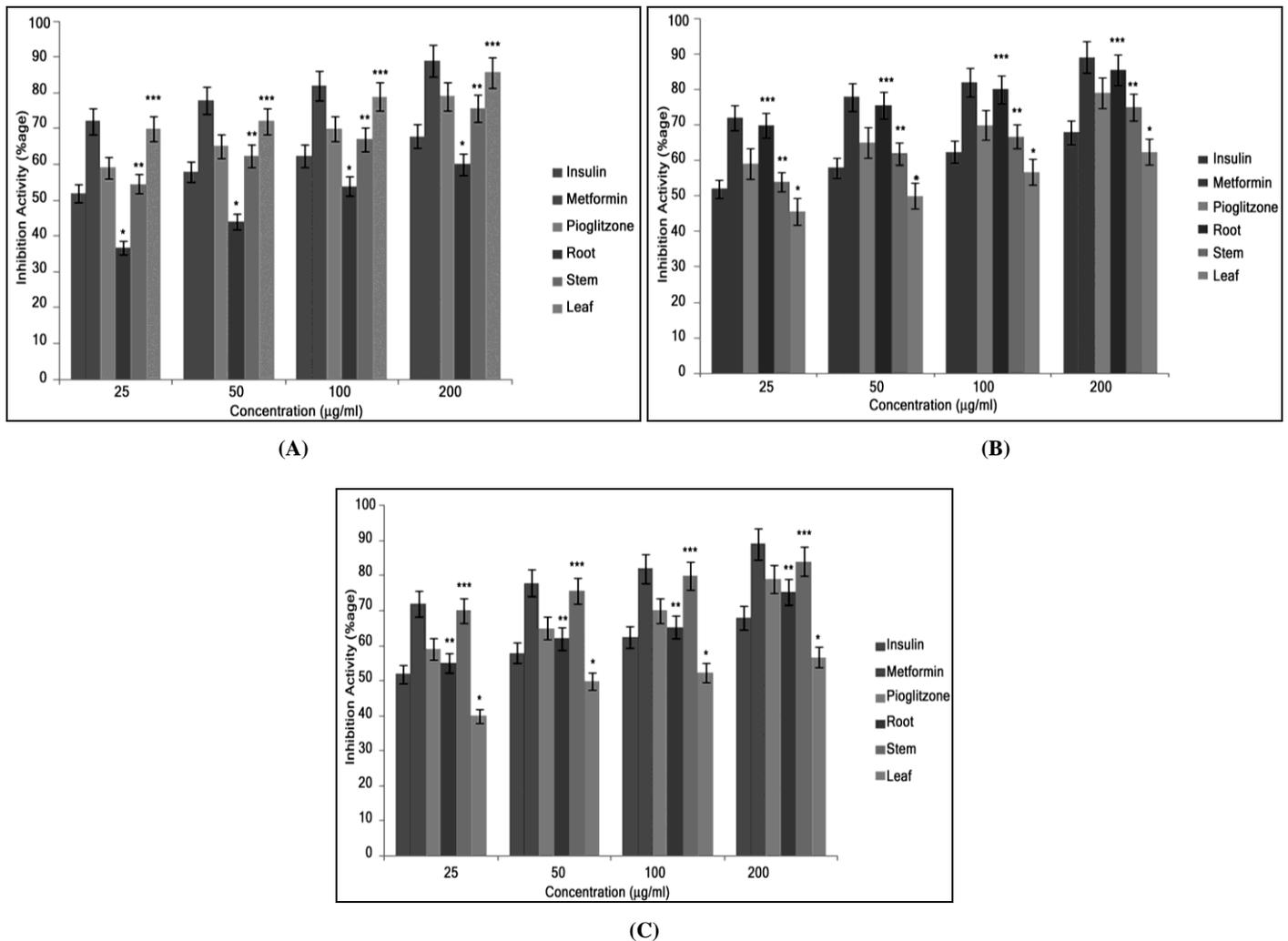


**Fig 2:** DPPH free radical scavenging activity (A) pet ether extract of leaf, stem and root (B) hydro ethanolic extract of leaf, stem and root (C) aqueous extract of leaf, stem and root of *Calotropis procera* vs. different anti-diabetic drugs. Mean values were significantly different from those for control incubations. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .

### 3.5 Superoxide radical scavenging activity

The ability to scavenge superoxide radical is shown in figure 3. The superoxide anion scavenging activity was estimated by measuring the reduction in rate of formation of formazan dye, which is blue in color. The resultant  $O_2^-$  reduces 2, 2-di-p-nitrophenyl-5, 5'-dphenyl-3, 3'-(3, 3-dimethyl-4, 4'-biphenylene)-ditetrazolium chloride (NBT) in to the formazan dye. From our investigation the superoxide radical scavenging activity order was reported as PEE of leaves (87%) > HEE of

leaves (63%) > AQE of leaves (57%). Pet-ether extracts of leaves had significantly higher radical scavenging capacity at concentrations 25, 50, 100, 200 µg/ml as compared to stem and root ( $p<0.001$ ) and the level of significance was almost equivalent to metformin. The aqueous extract of stem also exhibited significantly high radical scavenging capacity ( $p<0.001$ ). Ascorbic acid of equivalent concentration was used as reference control (Fig.3).



**Fig 3:** Superoxide radical scavenging activity (A) pet ether extract of leaf, stem and root (B) hydro ethanolic extract of leaf, stem and root (C) aqueous extract of leaf, stem and root of *Calotropis procera* vs. different anti-diabetic drugs. Mean values were significantly different from those for control incubations. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### 4. Discussion

Diabetes is a metabolic disorder which imbalances body's metabolic activity and is marked by increase in the reactive oxygen species production [19]. The production of antioxidant enzymes is however compromised in diabetics [20]. Plant extracts have been known to possess antioxidant properties to balance this antioxidant deficit [9].

Thus in this study different parts i.e. root, stem and leaf of *Calotropis procera* has been extracted to evaluate the presence/absence of phytochemical constituents and to study their antioxidant activity.

The antioxidant activity of the extracts was determined by the estimating the ability of extracts to scavenge DPPH radicals. DPPH (1, 1-diphenyl-2-picrylhydrazyl) is a stable free radical and can accept an electron or hydrogen radical to become a stable diamagnetic molecule [21]. Free radical scavenging is a recognized mechanism through which antioxidants prevent lipid peroxidation [22]. Our study concluded that the leaf extracts of *C. procera* possess DPPH scavenging activity as quantified by intensity of discoloration in concentration dependent manner. It also proved that the extracts are capable of donating an electron or hydrogen which could react with DPPH radical.

Superoxide anion, the reduced form of molecular oxygen is one of the precursors of hydroxyl or singlet oxygen species and many other free radicals, initiate directly oxidative damage in lipids, proteins, and DNA [23]. Figure 3 represents

the superoxide radical scavenging activity in comparison with different anti-diabetic drugs. Our study suggested that pet ether extracts of leaf and aqueous extracts of stem of *C. procera* had strong superoxide radical scavenging activity. Free radicals production leads to numerous metabolic disorders in human. Phytoconstituents has been implicated to control these disorders due to negative effects of synthetic antioxidants nowadays [24]. In recent years, the search for phytochemicals possessing antioxidant properties have been on the rise due to their potential use in the therapy of various chronic metabolic diseases [25]. Phytochemical screening of the extracts of *C. procera* revealed the presence of phenols, flavonoids, tannins, saponins which permits the use of plant in oxidative stress management. This investigation suggested that the leaf and stem extracts of *C. procera* had significant amount of these phytoconstituents. It has been confirmed that pharmacological effect of phenols is correlating with their antioxidant activities.

#### 5. Conclusion

It is beneficial to accept practice of natural antioxidant to prevent human health from the harmful effects of synthetic antioxidant. In this study, leaf, stem and root extracts of *C. procera* efficiently showed significant amount of phytoconstituents and the leaf extracts particularly exhibited significant antioxidant properties comparable with that of anti-diabetic drugs. Thus we can conclude that *C. procera*

extracts can be used as a source of natural antioxidants to control diabetes however further *in vitro* and *in vivo* experiments are needed to validate the candidature of the plant extract as a potent antidiabetic compound.

## 6. Abbreviations

PEE; pet ether extract; HEE; hydro ethanolic extract; AQE; aqueous extract; NBT; nitro blue tetrazolium.

## 7. Conflict of interest statement

The authors declare that they have no conflict of interest.

## 8. Acknowledgement

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