



## ***In vitro* antioxidant and PTP inhibitory activity of methanolic extract of *Anogeissus acuminata* leaf and bark.**

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### ABSTRACT

**Objective:** *Anogeissus acuminata* is a plant rich in tannins and flavonoids. Various parts of the plant are used in traditional medicine for diabetes mellitus and inflammatory conditions. In the present study, methanolic extracts of leaf and bark are evaluated for their *in vitro* antioxidant and PTP 1B inhibitory activity. **Methods:** *In vitro* antioxidant activity was analyzed by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, reducing power assay and TBA (thiobarbituric acid) assay, using vitamin C as a standard. Moreover, antidiabetic potential was evaluated using *in vitro* protein tyrosine phosphatase 1B (PTP1B) inhibition assay using Suramin as a standard. **Results:** IC<sub>50</sub> values for Vitamin C, leaf and bark of the plant in DPPH assay were  $45.51 \pm 6.657$ ,  $111.9 \pm 5.534$  and  $80.9 \pm 5.329$   $\mu\text{g/ml}$ , respectively. The extracts also exhibited significant reducing powers and inhibition of lipid peroxidation comparable to that by Vitamin C ( $P < 0.01$ ). Both extracts also demonstrated significant PTP 1B inhibitory activity with IC<sub>50</sub> values  $20.7 \pm 7.6$  and  $11.59 \pm 7.8$   $\mu\text{g/ml}$  for leaf and bark extracts respectively. **Conclusion:** Leaf and bark extracts of *Anogeissus acuminata* demonstrated potent *in vitro* anti-oxidant and PTP1B inhibitory activity.

**KEYWORDS:** *Anogeissus acuminata*, anti-oxidant, PTP inhibitory activity, anti diabetic

### INTRODUCTION

Protein Tyrosine Phosphatase 1B is a negative regulator of insulin and leptin receptor signaling. PTP1B dephosphorylates the activated insulin receptors or insulin receptor substrates (IRSs). PTP1B inhibition is a promising drug target for type 2 diabetes and obesity<sup>1</sup>. Many natural products have been identified as inhibitors of PTP1B enzyme. Moreover, phenolics have demonstrated potent PTP1B inhibitory activity<sup>2</sup>.

*Anogeissus acuminata* (AA), also known as *dhok* or *kardhai* belongs to the family, Combretaceae. Being a plant rich in phenolics like flavonoids and tannins, it is likely to have action on PTP1B enzyme. Hence, we attempted to assess the antioxidant potential and PTP1B inhibitory activity of AA. Parts of this plant are used in treatment of diabetes mellitus and various other inflammatory and painful conditions in India and Thailand<sup>3,4</sup>. However, it has not been evaluated for its action on enzyme protein tyrosine phosphatase. Therefore, aim of the present study is to provide a scientific evidence and explanation for its traditional use.

It is a well-known fact that oxidative stress plays a decisive role in pathogenesis of diabetes mellitus by affecting mitochondrial energy production and altering lipids, proteins and nucleic acids<sup>5</sup>. Oxidative stress also plays an important role in development of various complications of diabetes and progression of diabetes by causing beta cell necrosis<sup>6</sup>. WHO has recommended scientific evaluation of traditionally used antidiabetic plants<sup>7</sup>. A plant having hypoglycemic action combined with potent anti-oxidant profile will prove to be highly effective in improving therapeutic outcomes in diabetes patients. Therefore, we assessed both these activities of methanolic extracts of bark and leaves of AA.

### MATERIALS AND METHODS

#### Plant collection and extraction

Leaves and bark of AA were collected from North Gujarat, 24° 1' 42" N, 73° 2' 29" E, India in december, 2012. The material was dried in shade and coarsely powdered. Herbarium of the collected sample was submitted for authentication at NISCAIR, Delhi, India with provided reference no. NISCAIR/RHMD/consult/2013/2290/70. The leaf and bark powder was extracted with methanol using Soxhlet extractor, evaporated with rotary evaporator and stored at -20°C.

#### Materials

PTP1B inhibition colorimetric assay kit (ab139465) was purchased

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from Abchem (Cambridge, UK). The solvents used were of HPLC grade. L-Ascorbic acid, BHA (butylatedhydroxy anisole), 1, 1-diphenyl-2-picryl hydrazyl (DPPH) and other chemicals used were purchased from Sigma Chemical Co, St. Louis, MO, USA.

### Antioxidant Assay

The antioxidant activity of AA extracts was determined by different *in vitro* methods such as the DPPH free radical scavenging assay, Thiobarbituric acid and reducing power methods. All the assays were carried out in triplicate, and average values were considered.

### DPPH Radical Scavenging Activity

DPPH scavenging activity of the plant extracts was carried out according to the method of Gyamfi *et al*<sup>8</sup>. Methanolic solution of plant extracts (50 µl) at different concentrations (10 µg/ml-10 mg/ml) was mixed with 450 µl of tris HCl buffer (50 mM, pH 7.4). One milliliter 0.1 mM DPPH in methanol was added to the above mixture. The mixture was shaken vigorously and incubated for 30 min at room temperature. Absorbance of the resulting solution was measured at 517 nm UV-Visible Spectrophotometer. Methanolic solution of leaf or bark of AA was used as blank and DPPH methanolic solution served as control. The Vitamin C was used as a standard antioxidant in this method. Percentage of DPPH scavenging activity was determined as follows:

$$\% \text{ Inhibition} = \frac{(A_{\text{Control}} - A_{\text{test}}) \times 100}{A_{\text{Control}}}$$

Where  $A_{\text{Control}}$  = absorbance of control,  $A_{\text{test}}$  = absorbance of test

Decreased absorbance of the reaction mixture indicates stronger DPPH radical scavenging activity.

### Reducing Power Assay

One milliliter of methanolic solution of plant extracts (final concentration 1 to 50 µg/ml) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide  $[K_3Fe(CN)_6]$  (10 g/l). After 20 min incubation at 50°C, 2.5 ml of trichloroacetic acid (100 g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 20 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml  $FeCl_3$  (1 g/l). Absorbance of the resultant solution was measured at 700 nm in UV-Visible Spectrophotometer; 2.5 ml solution of ascorbic acid (concentration 1 to 50 µg/ml) and phosphate buffer were used as standard and control group, respectively. Methanolic solution of plant extracts was used as blank. Increased absorbance of the reaction mixture indicates stronger reducing power<sup>9</sup>.

### Thiobarbituric acid (TBA) method

The method of Ottolenghi<sup>10</sup> was used. Two ml of 20% trichloroacetic

acid and 2 ml of 0.67% 2-thiobarbituric acid was added to 1 ml of 20 µg/ml sample solution. The mixture was placed in a boiling water bath and, after cooling, was centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant was measured at 552 nm. Antioxidant activity was based on the absorbance observed at 14<sup>th</sup> day<sup>11</sup>.

### PTP1B inhibition Assay

The bark and leaf extracts of AA were evaluated for their PTP1B inhibitory activity in an *in vitro* assay. Suramin was used as positive control. IR5 Insulin receptor β residues 1142-1153, pY-1146 was used as PTP1B substrate. Assay was performed as per the instructions provided in kit. To each well of a 96 well plate, 85µl of assay buffer, 5 µl of (0.5 ng/µl) PTP 1B enzyme and 50 µl of 75 µM PTP1B substrate were added and incubated at 30 °C. After 30 min reaction was terminated by addition of 25 µl of provided red assay reagent and allowed to stand for 20 min before reading the absorbance at 620 nm on ELISA plate reader<sup>12</sup>.

### Statistical Analysis

Results are expressed as mean±SEM of three determinants. Comparison among the groups was tested by one-way ANOVA.  $P < 0.01$  values were considered significant. The curve-fitting program Prism 5 (GraphPad Software, San Diego, CA) was used to calculate IC50 values.

## RESULTS

### DPPH radical scavenging activity

DPPH radical scavenging activity is presented in Fig 1. Both leaf and bark extract showed significant radical scavenging activity as compared to Vitamin C with IC50 values  $111.9 \pm 5.534$ ,  $80.9 \pm 5.329$  and  $45.51 \pm 6.657$ µg/ml respectively.

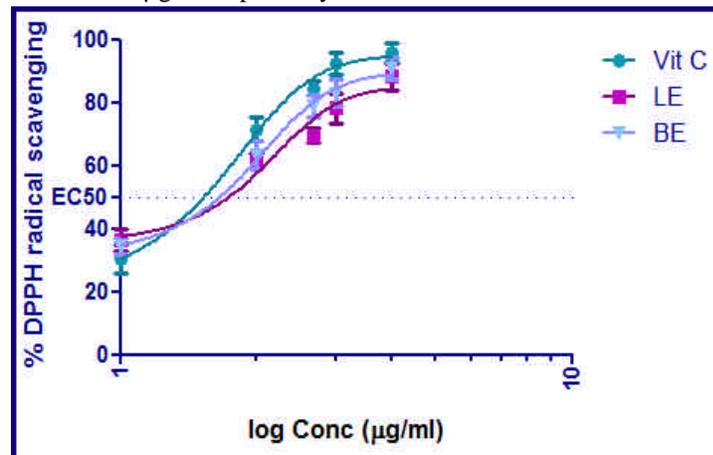
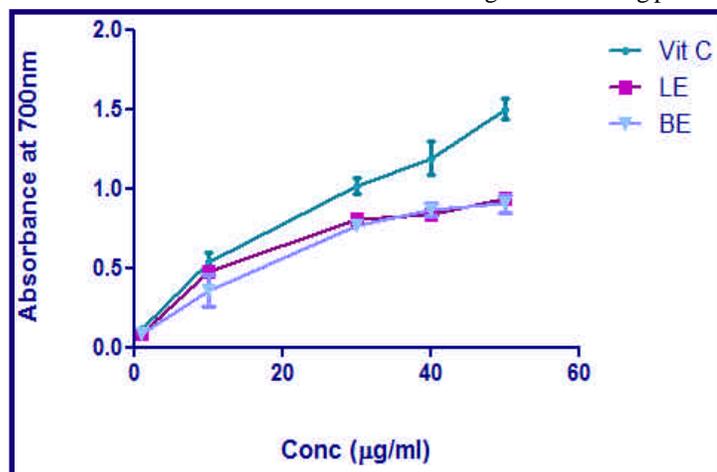


Fig. 1: Effect of methanolic extracts of leaf and bark of *Anogeissus acuminata* on DPPH radical scavenging activity. Values are expressed as mean ± standard deviation (n=3). Analyzed using GraphPad Prism 5.0, Non linear fit. Log (agonist) Vs Response (three parameters)

### Reducing Power Assay

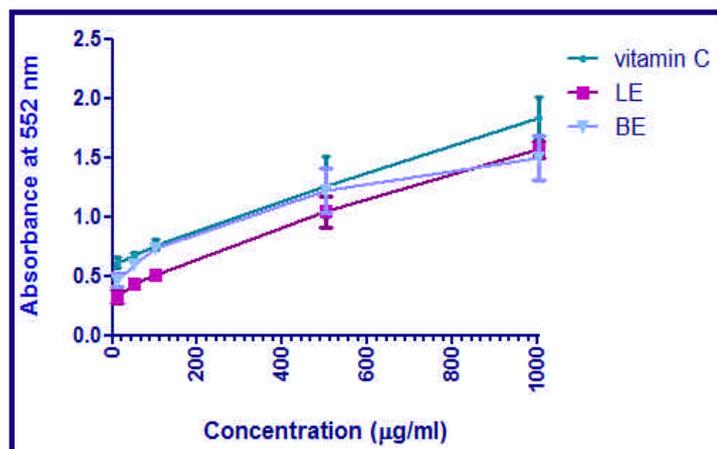
Both the extracts exhibited significant reducing power as demonstrated by the absorbances of reaction solutions (fig 2). Higher value of absorbance of the reaction mixture indicated greater reducing power.



**Fig. 2:** Effect of methanolic extract of leaf and bark of *Anogeissus acuminata* in reducing power assay. Values are expressed as mean  $\pm$  standard deviation (n=3).

### Thiobarbituric acid (TBA) method

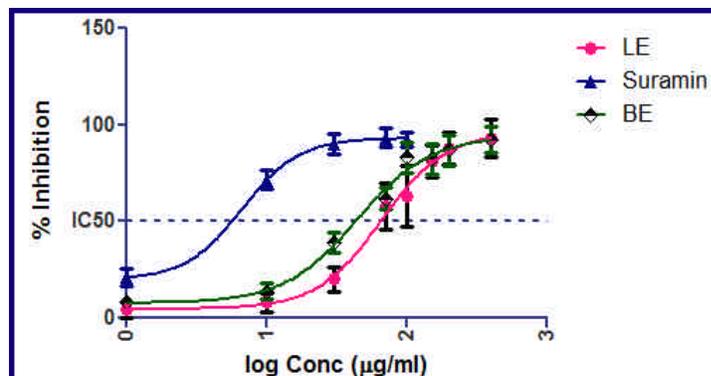
Results for TBA assay are presented in Fig 3. Both the extracts showed inhibition of lipid peroxidation as demonstrated by their lower absorbance values as compared to negative control. Both the extracts show comparable activity to Vitamin C ( $P > 0.05$ ).



**Fig 3:** Effect of methanolic extract of leaf and bark of *Anogeissus acuminata* in Thiobarbituric acid assay. Values are expressed as mean  $\pm$  standard deviation (n=3). Analyzed by two way ANOVA followed by Bonferroni Post test

### PTP1B inhibition Assay

Leaf as well as bark extracts demonstrated significant PTP1B inhibitory activity as depicted in Figure 4. The IC<sub>50</sub> values for Suramin, leaf and bark extract were 6.61, 65.60 and 43.78  $\mu\text{g/ml}$  respectively.



**Fig. 4:** PTP inhibition activity of *Anogeissus acuminata* Leaf Extract, Bark Extract. Analysed using GraphPad Prism 5.0; Non linear curve fit; log(inhibitor) vs. response - Variable slope (four parameters)

### DISCUSSION

Methanolic extracts of AA demonstrated potent anti-oxidant activity. The DPPH radical has an odd electron, which is responsible for absorbance maxima at 517 nm and deep purple color of DPPH free radical. Antioxidants scavenge DPPH radical by donating an electron to it, such reduced DPPH is colorless. Thus lower absorbance after incubation with sample indicates a strong antioxidant activity of the sample. The DPPH scavenging activity of both extracts is in agreement with those observed in previous studies<sup>13</sup>.

Reducing power assay involves the reduction of  $\text{Fe}^{3+}$ /ferricyanide complex to the ferrous form of Perl's Prussian blue. The Perl's Prussian blue formed has absorbance maxima at 700nm. It was observed that leaf and bark extracts of AA are strong reductants as depicted from the formation of more Perl's Prussian blue and higher absorbance at 700nm.

The TBA assay indicates the extent of lipid peroxidation in later stages of lipid peroxidation. Both extracts of plant demonstrate an ability to prevent lipid peroxidation comparable to that by vitamin C. Lipid peroxidation is an important factor leading to macrovascular and microvascular damage in long standing diabetes<sup>14</sup>. Ability to prevent lipid peroxidation may impart an consequential benefit to its antidiabetic action.

Though antidiabetic effect of AA was previously reported<sup>3, 15</sup>, there was no assessment of its possible mechanism of action. The plant extracts demonstrated good PTP1B inhibitory action. PTP1B is a downregulator of insulin signaling. Thus, inhibition of PTP1B may improve insulin sensitivity and glucose utilization by tissues.

### CONCLUSION

Methanolic extracts of AA leaf and bark demonstrated potent antioxi-

dant and PTP1B inhibitory activity in *in vitro* tests. This suggests that the plant may be beneficial in prevention of long-term complications of diabetes mellitus, which needs further investigation.

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