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Research Article

## EXTRACTION AND EVALUATION FOR ANTIOXIDANT ACTIVITY OF PLANT PLATINO ASIATICA PARTS

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**Abstract:**

*Plantago Asiatica has antioxidant activity against Azathioprine induced oxidative stress in rats by decreasing the oxidative stress biomarkers serum creatinine, serum urea in kidneys. Plantago Asiatica has antioxidant effect, elevated by measuring antioxidant enzymes. There is increase in superoxide dismutase in liver and kidney tissues in Azathioprine induced oxidative stress in rats. Plantago Asiatica has high scavenging activity against DPPH free radical generating system. Plantago Asiatica has nephroprotective effect against Azathioprine induced toxicity in kidneys by observing the histopathological changes in rat kidney tissues. Plantago Asiatica has many pharmacological activities like anticancer, antimicrobial, anti-inflammatory, analgesic, antiarthritic, antibacterial, anti-HIV, and antihelminthic activities.*

**Key Words:** *Plantago Asiatica, antioxidant activity.*

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## INTRODUCTION:

**Free radicals in Health and Disease:**-A free radical is defined as any molecular species that contains an unpaired electron in the atomic orbital (Halliwell and Gutteridge, 1999). Radicals are highly reactive that either donate an electron to or extract an electron from other molecules, and therefore, behave as oxidants or reductants. As a result of their high reactivity, most radicals have a very short half life (10-6 seconds or less) in biological systems (Halliwell and Gutteridge, 1999). The most important free radicals produced in the body are oxygen derivatives, particularly superoxide and the hydroxyl radical. Examples of free radicals and reactive oxygen species include: superoxide anion radical, hydroxyl radical, nitric oxide, thiyl radical, trichloromethyl radical, hypochlorite radical, hypochlorous acid, and also some potentially dangerous non-radicals such as hydrogen peroxide, singlet oxygen, hypochlorous acid and ozone. Radical production in the body occurs by both endogenous and environmental factors

### Antioxidants

An antioxidant is defined as: "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate" (Halliwell, 1995). The physiological role of antioxidants is to prevent damage to cellular constituents arising as a consequence of chemical reactions involving free radicals.

**An ideal antioxidant:** An ideal antioxidant should have the following attributes –

- No harmful physiological effects.
- Effective in low concentration. Fat-soluble.
- Carry-through effect.
- Not contribute an objectionable flavour, odour or colour to the food.
- No destruction during processing.
- Readily-available.
- Economical

### Nephrotoxicity

**Pathogenic mechanism of drug induced nephrotoxicity** Most drugs found to cause nephrotoxicity by one or more common pathogenic mechanisms. These include altered intraglomerular hemodynamics, tubular cell toxicity, inflammation, crystal nephropathy, rhabdomyolysis, and thrombotic microangiopathy.

### Mechanisms of drug induced renal damage:

- a) Free radical production
- b) Disturbance of renal tubule cell energy metabolism

- c) Disrupted cell calcium homeostasis
- d) Alteration of membrane phospholipid metabolism
- e) Disruption of cellular monovalent cation volume and pH dependant degradation
- f) Disruption of cytoskeleton
- g) Abnormalities of cell proteases
- h) Abnormalities of protein and nucleic acid synthesis
- i) Distruption of lysosomal function

### Aims and Objectives:-

The present study has been designed to achieve the following aims and objectives.

- To evaluate the antioxidant activity of *Plantago Asiatica* against azathioprine induced oxidative stress in rats.
- To estimate various oxidatative stress biomarkers like Urea, and Creatinine in plasma to assess the antioxidant activity of test compound.
- To assess the antioxidant capacity of test compound by estimating superoxide dismutase in kidney tissue homogenate.
- To evaluate the antioxidant activity of test compound by examining the histopathological protection against azathioprine induces oxidative stress to kidney.

## METHODOLOGY

### Collection and Authentication of Plant Material

The Aerial Parts of *P. Asiatica* were collected and authenticated

### Extraction of Plant Material

The plant is grinded in to a coarse powder with the help of suitable grinder.

### Cold Extraction (Methanol Extraction)<sup>38</sup>

In this work the cold extraction process was done with the help of methanol. About 200gms of powdered material was taken in a clean, flat bottomed glass container and soaked in 750 ml of methanol. The container with its contents were sealed and kept for period of 7 days accompanied by continuous shaking with the shaker. The whole mixture then went under a coarse filtration by a piece of a clean, white cotton wool.

### Evaporation of Solvent

The filtrates (methanol extract) obtained were evaporated using Rotary evaporator in a porcelain dish. They rendered a gummy concentrate of greenish black. The extract was kept in vaccum dissector for 7 days.

### % Yield value of Methanol Extract from Aerial Parts of *P. Asiatica* Plant

Powder taken for extraction = 200gm

Weight of the empty china dish = 53.70gm

Weight of the china dish with extract = 73.24gm

Weight of the extract obtained = (73.24-48.70) gm = 24.54 gm

% yield of methanol extract =

$(\text{weight of extract})/(\text{powder taken for extraction}) \times 100 = 24.54/200 \times 100 = 12.27 \%$ .

### *In Vitro* Method:

**DPPH scavenging activity procedure:** - DPPH radical scavenging activity was measured using the method of Cotellet *et al.*, with some modifications. 3 ml of reaction mixture containing 0.2 ml of DPPH (100  $\mu\text{M}$  in methanol) 2.8 ml of test solution, at various concentrations (5, 10, 20, 40, 80, 160 320  $\mu\text{g/ml}$ ) of the synthetic compound was incubated at 37°C for 30 min absorbance of the resulting solution was measured at 517 nm using Beckman model DU-40 spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control using the following equation:

$\% \text{ scavenging activity} = \frac{\text{absorbance of blank} - \text{absorbance of test}}{\text{Absorbance of blank}} \times 100$   
+  $\text{IC}_{50}$  will obtain from a plot between concentration of test compounds and % scavenging. Ascorbic acid is used as standard for comparison.

### *In Vivo* Method:

**Experimental animals:** 20 adult male albino rats weighing 140-160g were used for the study were procured. They were housed in polypropylene cages and were maintained at room temperature of 23°C  $\pm$  2°C and relative humidity 50%. They were maintained in 12h: 12hr light: dark cycle throughout the period of acclimatization and experimental study. Animals were provided with standard rodent pellet diet. Food and water was allowed *ad libitum*.

### Acute toxicity study of *Plantago Asiatica* formulation (As per OECD guide Lines number: 423):

The Acute Toxicity Studies was performed using female rats as per OECD Guideline No.423 (short term toxicity). The compound found to be non toxic and safe up to 2000mg/kg body weight by oral route. After 48hr animals were well tolerated. There was no mortality and no signs of toxicity. So two doses are selected i.e. 100mg/kg and 200mg/kg are selected as low and high dose.

### Induction procedure:-

#### Induction of oxidative stress:-

3mg/ml of Azathioprine solution was given through oral to all the group of animals and the samples were collected from the animals through retro-orbital plexus root and the kidney bio marker parameters were estimated like Creatinine, and Urea.

#### Experimental design:-

The animals were assigned to five groups, each group containing six rats:

**Group I:** Rats were orally administered with normal saline for 21days as the normal control.

**Group II:** Rats were orally administered with Azathioprine (20mg/kg) for 21days.

**Group III:** Rats were treated with azathioprine (20mg/kg) and treated with test compound (100mg/kg) by oral for 21days.

**Group IV:** Rats were treated with azathioprine (20mg/kg) and treated with test compound (200mg/kg) by oral for 21days.

**Group V:** Rats were treated with azathioprine (20mg/kg) and treated with ascorbic acid (10mg/kg) by oral for 21days.

#### Collection of blood samples and organs:

Blood samples were collected from all the groups of animals 24hours after the 21st day of treatment through puncture of retro orbital plexus and were centrifuged at 3000 revolutions per minute (RPM) for 15 minutes. Serum was separated and stored at -20°C and used for estimating urea and creatinine levels. Rats were killed by over anesthesia, a midline abdominal incision is made to open up the abdominal cavity and access the liver and kidney. The liver and right kidney are removed rapidly and washed with saline, then fixed quickly in formaldehyde. The liver and left kidney were homogenized in 0.25 M cold sucrose solution and centrifuged at 5000 rpm for five minutes. The supernatant which is store at -20°C used for the quantitative estimation of superoxide dismutase within 48hours by using uv spectrophotometer [UV method].

#### Estimation of biochemical parameters:-

The following are the biochemical parameters estimated to evaluate the effect of the test materials against the experimentally induced oxidative stress in rats. They are SOD, Urea and Creatinine.

#### Estimation of Superoxide Dismutase (SOD)

Superoxide dismutases are the enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen.

**Estimation of serum Creatinine levels****Clinical Significance:**

Creatinine is the catabolic product of creatinine phosphate, which is used by the skeletal muscle. The daily production depends on the muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow and were decreased in muscular dystrophy.

**Principle:** Creatinine + Sodium Picrate  $\rightarrow$  creatinine picrate-complex (Yellow-orange)

Intensity of the color formed is directly proportional to the amount of creatinine present in the sample.

**Methodology:** Modified Jaffers Kinetic method

**Reagents:**

Working reagent: Add 2.5 ml of reagent 2 to 205ml of reagent 1

**Procedure:**

To 1 ml of working reagent, 50 $\mu$ l of serum or the creatinine standard were added and mixed. Read the absorbance after 30 sec ( $A_0$ ) and 90 sec ( $A_1$ ) using UV spectrophotometer at 520 nm and determine the  $\Delta A$  for standard (S) and test (T). Calculate the serum creatinine in mg/dl in the test sample using the following equation.

Serum creatinine in mg/dl =  $(\Delta A_T / \Delta A_S) \times 2$ .

**Estimation of serum urea levels****Clinical significance:**

Urea is the major metabolite product of protein catabolism. The biosynthesis of urea from ammonia is exclusively carried out by hepatic enzymes. More than 90% of urea is excreted through the kidneys, with the remainder excreted through the gastrointestinal tract or skin. Blood urea concentrations can be increased by numerous factors linked to prerenal causes (increased protein catabolism, as in hemorrhage into gastrointestinal tract, shock, some chronic liver diseases) or renal/postrenal causes (acute or chronic renal diseases, post renal obstruction to urine flow). Uremia is also increased by high-protein diet, state of dehydration, muscle wasting (as in starvation). The determination of urea rate is used together with the determination of creatinine rate to discriminate between prerenal (normal creatinine) and renal/postrenal (increased creatinine) disorders.

**Principle**

Urea in an acidic medium condenses with diacetyl monoxime at 100°C to form a red coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

100° C

Urea + DAM  $\rightarrow$  Red coloured complex

**Reagent**

1. Urea reagent
2. Acid reagent
3. DAM reagent
4. Urea standard (40 mg/dl)

**Procedure**

The entire reagents were brought to the room temperature before using the test. Undiluted serum sample was used in this method. Taken 3 set of test tubes and marked as Blank, Standard and Test. 0.001ml of serum sample was taken in test tube, 0.01ml of urea standard reagent in standard test tube, 0.01ml of distilled water was added to the Blank test tube. Added 1.0ml of urea reagent, acid reagent and DAM reagent to all the test tubes. These solutions were mixed well and kept in boiling water bath (100°C) for 10 minutes and cool in running tap water. Absorbance was read at 520 nm a reagent blank.

**Normal value:**

Serum Urea: 15-40 mg /dl.Dam Method

**Table 1: Assay procedure of serum urea**

Test tube Content	Blank (ml)	Standard (ml)	Test (ml)
Serum Sample	-	-	0.01
Urea Standard	-	0.01	-
Distilled Water	0.01	-	-
Urea Reagent	1.0	1.0	1.0
DAM Reagent	1.0	1.0	1.0
Acid Reagent	1.0	1.0	1.0

Heated in boiling water bath for 10 minutes. Read at 520nm using green filter.

**Calculation**

Serum Urea =  $\frac{\text{Absorbance test}}{\text{Absorbance of standard}} \times 40\text{mg/dl}$

**Histopathological examination:**

The animals were then dissected, the livers were from all groups carefully removed, washed with 0.9% saline solution and preserved in formalin solution (10% formaldehyde) for histopathological studies (Hesham A et al., 2010).

**Statistical analysis:**

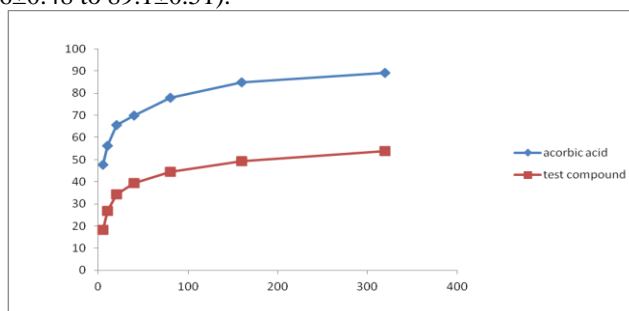
All the values were expressed as mean  $\pm$  standard deviation (S.D). Statistical comparisons between different groups will be done by using one way analysis of variance (ANOVA) followed by dunnett's test. P <0.05 will be considered as statistically significant.

**RESULTS AND DISCUSSION:****Table 2: *In vitro* Evaluation of Antioxidant Activity of *Plantago Asiatica* Dpph Radical Scavenging Activity:**

Concentrations of test compound and ascorbic acid ( $\mu\text{g/ml}$ )	Percentage inhibition of DPPH radical ( $\text{IC}_{50}$ )	
	<i>Plantago Asiatica</i> (EEPA)	Ascorbic acid
5	18.2 $\pm$ 0.81	47.6 $\pm$ 0.48
10	26.7 $\pm$ 0.51	56.15 $\pm$ 0.65
20	34.2 $\pm$ 1.15	65.6 $\pm$ 0.48
40	39.3 $\pm$ 0.47	70 $\pm$ 1.33
80	44.6 $\pm$ 0.77	77.8 $\pm$ 0.82
160	49.2 $\pm$ 0.68	84.9 $\pm$ 1.1
320	53.5 $\pm$ 0.90	89.1 $\pm$ 0.51

**Concentration dependent percentage inhibition of DPPH radical by various concentrations of test compound and ascorbic acid**

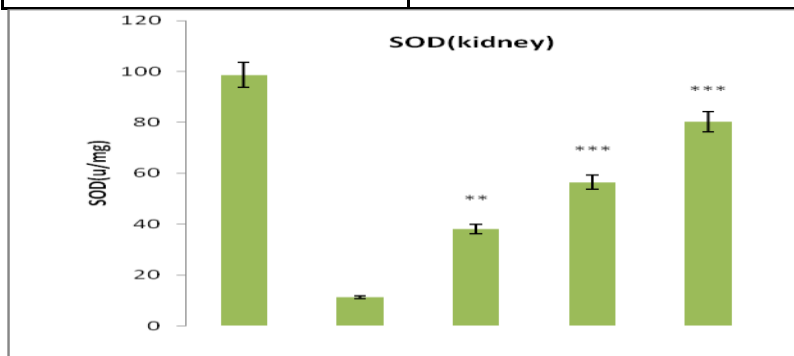
The test compounds have been reported to show high scavenging activity against the DPPH free radical generating system. The antiradical activity of test compound and ascorbic acid against DPPH was shown in Table and the  $\text{IC}_{50}$  values were found to be as 18.2 $\pm$ 0.81, to 53.5 $\pm$ 0.9 increased with respectively concentrations with that of reference standard, ascorbic acid (47.6 $\pm$ 0.48 to 89.1 $\pm$ 0.51).

**In vitro concentration dependent percentage inhibition of DPPH radical by EEPA and ascorbic acid**

The results clearly indicate the free radical scavenging activity of test compound in vitro and this activity comparable with that of standard drug ascorbic acid.

**Table 3: Superoxide dismutase levels in kidney tissue homogenate**

Group	SOD(U/mg) in kidney
Normal group	98.6 $\pm$ 0.95
Toxic control (20mg/kg)	11.3 $\pm$ 0.71
EEPA low dose(100mg/kg)	38.07 $\pm$ 0.52**
EEPA high dose (30mg/kg)	56.46 $\pm$ 1.08***
Standard ascorbic acid(10mg/kg)	80.2 $\pm$ 0.84***

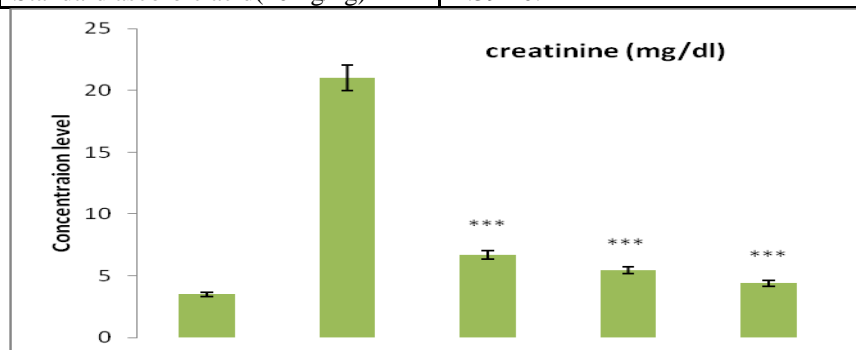


All the values are expressed as mean  $\pm$ SD (n=6); \*\* indicates  $p < 0.001$ , \*\*\* indicates  $p < 0.0001$  vs toxic control.

## Serum Creatinine:

Table 4: Effects of EEPA on serum creatinine levels in rats treated with azathioprine

Groups name	Creatinine (mg/dl)
Normal group	3.5± 0.32
Toxic control (20mg/kg)	21.05± 0.69
EEPA low dose(100mg/kg)	6.68± 0.27***
EEPA high dose (200mg/kg)	5.45± 0.15***
Standard ascorbic acid(10mg/kg)	4.39± 0.11***

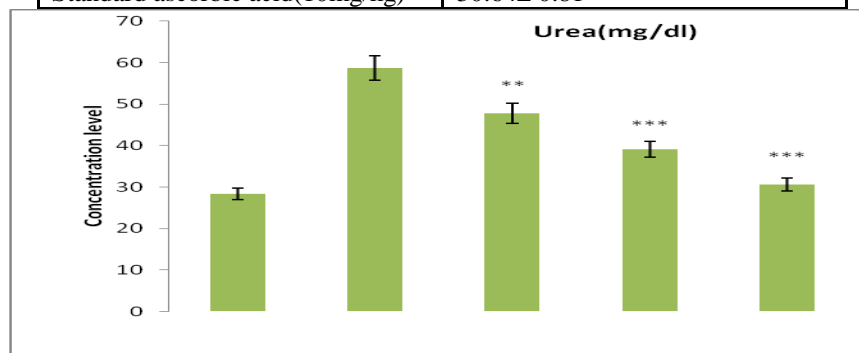


All the values of mean  $\pm$ SD; n= 6, \*\*\* indicates  $p<0.0001$  vs toxic control.

The above table shows the effect of test compound on serum creatinine levels in rats intoxicated AZP. After 21days treatment with AZP, the toxic control group shows  $21.05 \pm 0.69$  mg/dl increased compared with normal control group. Compared the test compound with toxic control group, at low dose of test compound serum creatinine level was decreased that is  $6.68 \pm 0.27$ mg/dl, has shown significance (\*\* $p<0.0001$ ) and at high dose of test compound serum creatinine level was decreased that is  $5.45 \pm 0.15$ mg/dl, has shown significance (\*\* $p<0.0001$ ). On treatment of standard ascorbic acid shows serum creatinine level  $4.39 \pm 0.11$ , has shown significance (\*\* $p<0.0001$ ).

Table 5: Effects of EEPA on serum urea levels in rats treated with azathioprine

Group name	Urea (mg/dl)
Normal group	28.4± 0.62
Toxic control (20mg/kg)	58.7± 0.60
EEPA low dose(100mg/kg)	47.8± 0.63**
EEPA high dose (200mg/kg)	39.1± 0.44***
Standard ascorbic acid(10mg/kg)	30.64± 0.81***



All the values of mean  $\pm$ SD; n= 6, \*\* indicates  $p<0.001$ , \*\*\* indicates  $p<0.0001$  vs toxic control.

After 21days treatment of all groups, the normal group shows serum urea level in normal range that is  $28.4 \pm 0.62$  mg/dl. The toxic control group shows serum urea level  $58.7 \pm 0.60$ , increased compared to the normal group. The test compound, at low dose shows serum urea level  $47.8 \pm 0.63$ , decreased compared to the toxic control group has shown significance (\*\* $p<0.001$ ) and at high dose shows serum urea level  $39.1 \pm 0.44$ , decreased compared to the toxic control group has shown significance (\*\* $p<0.0001$ ). On treatment of standard ascorbic acid shows serum urea level  $30.64 \pm 0.81$ , has shown significance (\*\* $p<0.0001$ ).

**CONCLUSION:**

On the basis of our findings, it may be worthy to suggest that

*Plantago Asiatica* has antioxidant activity against Azathioprine induced oxidative stress in rats by decreasing the oxidative stress biomarkers serum creatinine, serum urea in kidneys.

*Plantago Asiatica* has antioxidant effect, elevated by measuring antioxidant enzymes. There is increase in superoxide dismutase in liver and kidney tissues in Azathioprine induced oxidative stress in rats.

*Plantago Asiatica* has high scavenging activity against DPPH free radical generating system.

*Plantago Asiatica* has nephroprotective effect against Azathioprine induced toxicity in kidneys by observing the histopathological changes in rat kidney tissues.

*Plantago Asiatica* has many pharmacological activities like anticancer, antimicrobial, anti-inflammatory, analgesic, antiarthritic, antibacterial, anti-HIV, and antihelmintic activities.

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