Antioxidant Activity of *Plantago asiatica* against Azathioprine Induced Oxidative Stress in Rats

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**Abstract:** Through a decrease in the kidneys’ oxidative damage indicators, serum creatinine and serum urea, *Plantago asiatica* exhibits oxidation inhibitor action against oxidative stress generated by Azathioprine. Antioxidant enzyme measurements showed increased effects of *Plantago asiatica*. In rats exposed to azathioprine-induced oxidative stress, there is amplification in superoxide dismutase in the kidney tissues. The DPPH free radical producing mechanism is highly scavenged by *Plantago asiatica*. *Plantago asiatica* has nephroprotective action against Azathioprine-provoked toxicity in kidneys. The pharmacological properties of *Plantago asiatica* include anti-inflammatory, anti-tumour, anti-arthritic, antibacterial, anti-HIV, anti-cancer, and antihelmintic.

**Keywords:** *Plantago asiatica*, Azathioprine, Antioxidant, Serum creatinine, Serum urea, Superoxide dismutase

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**Introduction**

Any chemical entity having an unbounded electron in its atomic orbital is referred to as a free radical (Halliwell, 1999). Extremely reactive compounds, known as radicals, can behave as oxidants or reductants by either giving or taking electrons from other molecules. Since most radicals are highly reactive, their half-lives in biological systems are quite short (10–6 seconds or less) (Halliwell, 1999). Superoxide and the hydroxyl radical are two of the most significant free radicals that the body produces. An imbalance between the generation of free radicals and antioxidant defences is known as oxidative stress, and it can lead to detriment to a variety of molecular species, such as proteins, lipids, and nucleic acids. Lipid peroxidation, which is a process that is typically carried out by lipoprotein particles or membranes, produces an extensive variety of compounds,
including conjugated dienes, alkanes and alkenes, short chain aldehydes like malondialdehyde or 4-hydroxynonenal, and various hydroxides and hydroperoxides (Esterbauer, 1996). Similar to how oxidative injury to nucleic acids and proteins modifies nucleotides or amino acids, it also produces a range of particular damage products (Griffiths et al., 2002). Such oxidative damage may also result in cellular glitch and play a role in the pathogenesis of numerous diseases.

Many degenerative diseases, including diabetes, cancer, Alzheimer’s disease, neurological disorders, and aging, have been linked to oxidative stress (Griffiths et al., 2002). Furthermore, they have an impact on physical activity, stress, and acute illnesses such trauma, stroke, and infection (Scalbert et al., 2005).

Antioxidants are thought to be useful in reducing or delaying the occurrence of free radicals, as they are thought to be causally involved in the disease state. In fact, the idea that nutritional antioxidant status is inversely correlated with the incidence of free radical-mediated disorders is highly supported by studies conducted at the cellular, tissue, and whole animal levels moreover by epidemiological research (Glantzounis et al., 2005; Poeggeler, 2005).

**Antioxidants:**

All substances that considerably delay or prevent the oxidation of an oxidisable substrate when present at lower quantities are considered antioxidants (Halliwell et al., 1995). Preventing harm to cellular components emanating from chemical processes involving free radicals is the physiological function of antioxidants.

**Antioxidant Synergy:**

Antioxidant combinations work well together than when they are taken separately (Liu, 2004). Effectiveness is enhanced by combined interaction in a number of ways, including: vitamins E and C work better together in a lipid oxidation system than they do separately because ascorbate can diminish Vitamin E. Synergy is also known as co-antioxidants in biological samples.

The whole antioxidant defence system of living systems relies on antioxidant synergy (Closa and Folch-Puy, 2004).

**Antioxidant defence system:**

A wide spectrum of antioxidant defences have developed to shield the cell from harm caused by free radicals because radicals have the ability to react indiscriminately, damaging nearly every component of the cell (Nordberg and Arnér, 2001). Transition metal binding proteins, non-enzymatic antioxidants, and antioxidant enzymes comprise the three primary categories of cellular antioxidants (Halliwell, 1999).

**Plantago asiatica:**

Asian pharmacopoeia has long prescribed *Plantago asiatica* to cure liver problems. Additionally, it has been used to treat bladder and urinary tract inflammation as well as gastrointestinal issues. There is a slight laxative effect to its seeds. The plant’s leaves are utilized in a lot of Japanese recipes, particularly soups. *Plantago* species have been utilized as herbal medicines since ancient times (Closa and Folch-Puy, 2004). The herb has anti-inflammatory, anti-histamine, anti-microbial, anti-toxic, demulcent chest decongestant, haemostatic, and fluid eliminating properties. An herbal paste of the leaves applied externally helps with minor wounds, boils, rashes from poison ivy, and insect bites. It is even said in mythology to be able to heal snakebite. Taken internally as a tea, tincture, or syrup, it helps with bronchitis and coughing. Sometimes, the broad-leaved types are used as a leaf vegetable in salads, green sauce, and other dishes (Fraga-Corral et al., 2021). The husks of plantains, particularly *P. psyllium*, which can be employed in popular over-the-counter bulk laxatives and fiber supplements like Metamucil, expand and become mucilaginous when placed in water. Constipation, irritable bowel syndrome, diverticular disease, and dietary fibre supplementation can all benefit from *P. psyllium* seed. People have eaten plantains since prehistoric times. The usage of this species as food since the Milling stone Horizon, for instance, has been
shown by archaeological discoveries along California's Central Coast. In addition to drinking enough water, psyllium supplements are usually taken as powder (Dawid-Pać, 2013). Certain individuals manage increased cholesterol by taking a daily intake of at least 7 grams together with sufficient fluids (juice, water). Constipation can be treated with a variety of psyllium preparations. About 3.5 g taken as dual daily doses is the standard dosage. Moreover, psyllium can be found in a number of prepared foods. By grinding off the husk, one can produce mucilage from Desert Indian wheat (Plantago ovate). Isabgol, a laxative used to treat constipation and irregular bowel syndrome, is a popular brand name for this mucilage, usually referred to as psyllium. It is utilized for a variety of intestinal issues as an indigenous Unani and Ayurvedic remedy (Garg, 2021). Plantago major leaves have antiseptic qualities and are used as a folk treatment in Serbia, Romania, and Bulgaria to prevent infection from cuts and scrapes. The leaves were traditionally applied topically to sores emerging from friction (such as those induced by tight shoes, etc.) and to relieve mosquito nibble in both eastern and western Westphalia in Slovenia and other Central European locations (Attaluri et al., 2011). Plantains may also have a role in reducing enteric methane from ruminants because of their natural chemicals (such as condensed tannins, which are about 14 g/kg of dry matter) that alter the acetate-propionate ratio in the rumen, a key mechanism limiting methanogenesis. Because of agronomic challenges, this is currently not a feasible solution on any meaningful scale (Ye, 2011).

In this study serum creatinine and serum urea were measured in kidney of rats exposed to azathioprine and evaluated the role Plantago asiatica in nephrotoxicity of rats.

**Materials and Methods**

The following are agents or compounds utilized in the present investigation. Drugs and chemicals source are-- Azathioprine: RPG Life sciences Pvt, Ltd, Hyderabad; Ascorbic acid: Finar chemicals, Ahmadabad, India; DPPH: Sigma Aldrich, USA; Formaldehyde: Finar chemicals, Ahmadabad, India; Normal saline: Claris otsuka limited, Ahmedabad, India; Sodium citrate: Finar chemicals, Ahmadabad, India; Dipotassium hydrogen phosphate: Merck Pvt, Ltd, Mumbai, India; Potassium dihydrogen phosphate: Merck Pvt, Ltd, Mumbai, India; Diethyl ether: Molychem, Mumbai, India; Chloroform: Finar chemicals, Ahmadabad, India; O-dianisidine: Sigma Aldrich, USA; Ethanol: Merck Pvt, Ltd , Mumbai, India; Methanol: Merck Pvt, Ltd, Mumbai, India; Riboflavin: Sigma, st.louis, USA; Kits used: Urea: Excel diagnostics Pvt, Ltd, Hyd, India; Creatinine: Excel diagnostics Pvt, Ltd, Hyd, India.

**Plant description:**

Kingdom: Plantae; Order: Lamiales; Family: Plantaginaceae; Genus: Plantago; Species: asiatica; Common name: Arnoglossum.

**Collection of plants and preparation of extract:**

The aerial structures of *P. asiatica* were collected. The plant was ground into a coarse powder with the help of suitable grinder. Methanol was utilized to facilitate the cold extraction process. A clean glass container with a flat bottom was filled with about 200 g of powdered material, which was then steeped in 750 ml of methanol. The shaker was used to continuously shake the container and its contents for duration of seven days. After then, a piece of clean, white cotton wool was used to coarsely filter the entire mixture (Cardoso-Gutierrez et al., 2021). The resulting filtrates, or methanol extract, were evaporated in a porcelain dish using rotary evaporator. They produced a gooey, blackish-green concentration. For seven days, the extract was stored in a vacuum desiccator (Saikia and Robinson, 2018). % yield value of methanol extract from aerial parts of *P. asiatica* plant was 12.27%.

**In vitro method:**

**Procedure for measuring DPPH radical scavenging activity:**

This method was modified and utilized to measure
DPPH radical scavenging activity. 0.2 ml of DPPH (100 μM in 3 ml of reaction mixture methanol) and 2.8 ml of test solution at different concentrations (5, 10, 20, 40, 80, 160, 320 μg/ml) of the synthesized chemical was incubated at 37°C for about 30 min. The Beckman model DU-40 spectrophotometer was used to determine the absorbance of the resultant solution at 517 nm. By utilizing the following formula to compare the test and control findings, the percentage inhibition of DPPH radical was determined: A plot between the concentration of test compounds and the percentage of scavenging will yield the following formula: % scavenging activity = absorbance of blank – absorbance of test/absorbance of blank x 100 + IC_{50}. Ascorbic acid is used as a standard for comparison (Ruiz et al., 2013).

In vivo method:

Experimental animals:

Twenty male albino matured rats (140-160 g b wt.) were kept in polypropylene cages at temperature of 23°C ± 2°C, relative humidity 50% and 12 h light-dark cycle for the duration of the experimental investigation and acclimation. The animals were fed a regular diet of rodent pellets. Water and food were available at all times (Baliyan et al., 2022).

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

As per OECD Guideline No. 423 (short term toxicity), the Acute Toxicity Studies were conducted on female rats. The substance was discovered to be safe and nontoxic at oral doses up to 2000 mg/kg b wt. The animals were tolerated well after 48 hours. Both death and toxicological symptoms were absent. As a result, two doses-100 mg/kg and 200 mg/kg-are chosen as the low and high doses (Albus, 2012).

Induction procedure for oxidative stress:

All of the animals in the group received an oral dose of 3 mg/ml of Azathioprine solution. Samplings were taken from the animals using a retro-orbital plexus root, and kidney biomarkers such as urea and creatinine were estimated.

Experimental design:

The animals were divided into five groups (n = 6 in each group):

Group I: Standard control, rats were given normal saline orally for duration of 21 days.

Group II: For 21 days, rats received 20 mg/kg of azathioprine orally.

Group III: Test and azathioprine (20 mg/kg) were administered to rats. Substance orally for 21 days at a dose of 100 mg/kg.

Group IV: For 21 routine days, rats were given 200 mg/kg of the test chemical or 20 mg/kg of azathioprine orally.

Group V: For 21 days, rats received oral treatments of ascorbic acid (10 mg/kg) and azathioprine (20 mg/kg).

Collection of blood samples and organs:

Using a retro-orbital plexus puncture, blood samples were taken from each group 24 h following the 21st day of therapy. The samples were then centrifuged for 15 min at 3000 rpm. To estimate the levels of urea and creatinine, serum was isolated, frozen at -20°C, and utilized. Overanesthesia caused the death of the rats; to access the kidney, a midline abdominal incision was performed. The right kidneys were swiftly removed, cleaned in saline, and then fixed in formaldehyde. Following homogenization in a 0.25 M cold sucrose solution, the left kidneys were centrifuged for 5 min at 5000 rpm. Superoxide dismutase was quantitatively estimated within 48 h using a UV spectrophotometer (UV technique) utilizing the supernatant, which was stored at -20°C (Albus, 2012).

Estimation of biochemical parameters:

The biochemical aspects listed below were determined in order to assess how well the test materials protected the rats from experimentally induced oxidative stress. They are creatinine, urea, and SOD.
Table 1: Concentration dependent percentage inhibition of DPPH radical by various concentrations of test compound and ascorbic acid

<table>
<thead>
<tr>
<th>Concentrations of test compound and ascorbic acid (µg/ml)</th>
<th>Percentage inhibition of DPPH radical (IC₅₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plantago asiatica (EEPA)</td>
</tr>
<tr>
<td>5</td>
<td>20.2</td>
</tr>
<tr>
<td>10</td>
<td>26.5</td>
</tr>
<tr>
<td>20</td>
<td>33.2</td>
</tr>
<tr>
<td>40</td>
<td>38.2</td>
</tr>
<tr>
<td>80</td>
<td>42.2</td>
</tr>
<tr>
<td>160</td>
<td>47.8</td>
</tr>
<tr>
<td>320</td>
<td>52.8</td>
</tr>
</tbody>
</table>

Estimation of Superoxide Dismutase (SOD) (Parasuraman et al., 2010; Misra and Fridovich, 1977), creatinine (Vidyasagar et al., 2004) and urea (Williamson and New, 2014) were performed.

Statistical analysis:
Data are expressed as mean ± standard deviation (S.D.). One way analysis of variance (ANOVA) was used for statistical comparisons between groups, followed by Dennett’s test. P values less than 0.05 are regarded as statistically significant.

Results and Discussion

In vitro evaluation of antioxidant activity of Plantago asiatica DPPH radical scavenging activity:
The test compounds showed high scavenging activity against the DPPH free radical generating system. The antiradical activity of test compound and ascorbic acid against DPPH is shown in Table 1. IC₅₀ values were found to be between 20.2 and 52.8 which increased with respective concentrations and for reference standard, ascorbic acid (46.5 to 88.5). The results clearly indicate the free radical scavenging activity of test compounds in vitro and this activity is comparable with that of standard drug ascorbic acid (Fig. 1).

In vivo studies:
Evaluation of antioxidant activity using Azathioprine induced oxidative stress in rats:
Superoxide dismutase: Superoxide dismutase is a class of enzymes that catalyze the disputation of superoxide into oxygen and hydrogen peroxide. It is an important antioxidant defence in nearly all cells exposed to oxygen. Superoxide dismutase activity was estimated in tissue homogenate with help of pure bovine superoxide dismutase standard. The values are shown in Tables 2, 3 and Figures 2, 3.

In this study, we found that 20 mg/kg dose of azathioprine (AZP) causes significant (p<0.001) decrease in superoxide dismutase levels. This reduction indicates that oxidative stress and toxicity is produced with azathioprine. Post treatment with a test compound at the dose of 100 mg/kg and 200 mg/kg after a 20 mg/kg dose of azathioprine administration, showed a significant (p<0.001, p<0.0001) dose dependent increase in levels compared to the toxic control group (Fig. 3).

Serum creatinine:
Table 4 shows the effect of test compound on serum creatinine levels in rats intoxicated AZP.
Fig. 1: *In vitro* concentration dependent percentage inhibition of DPPH radical by EEPA and ascorbic acid.

Table 2: Standard graph values of superoxide dismutase

<table>
<thead>
<tr>
<th>SOD (µU)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0.012</td>
</tr>
<tr>
<td>3000</td>
<td>0.015</td>
</tr>
<tr>
<td>10000</td>
<td>0.029</td>
</tr>
<tr>
<td>30000</td>
<td>0.058</td>
</tr>
<tr>
<td>100000</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Fig. 2: Standard graph of superoxide dismutase.
Table 3: Superoxide dismutase levels in kidney tissue homogenate

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD(U/mg) in kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal group</td>
<td>96.6±0.93</td>
</tr>
<tr>
<td>Toxic control (20 mg/kg)</td>
<td>10.3±0.71</td>
</tr>
<tr>
<td>EEPA low dose (100 mg/kg)</td>
<td>36.07±0.54**</td>
</tr>
<tr>
<td>EEPA high dose (30 mg/kg)</td>
<td>54.46±1.12***</td>
</tr>
<tr>
<td>Standard ascorbic acid (10 mg/kg)</td>
<td>81.2±0.86***</td>
</tr>
</tbody>
</table>

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

Fig. 3: Effect of EEPA on superoxide dismutase levels in kidney tissue homogenate in rats treated with AZP.

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

<table>
<thead>
<tr>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal group</td>
</tr>
<tr>
<td>Toxic control (20 mg/kg)</td>
</tr>
<tr>
<td>EEPA low dose (100 mg/kg)</td>
</tr>
<tr>
<td>EEPA high dose (200 mg/kg)</td>
</tr>
<tr>
<td>Standard ascorbic acid (10 mg/kg)</td>
</tr>
</tbody>
</table>

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

After 21 days of treatment with AZP, the toxic control group showed an increase as compared with the normal control group. Compared the test compound with toxic control group, at low and high dose of test compound serum creatinine level was significantly decreased.

**Serum urea:**

After 21 days treatment, the toxic control group showed increased serum urea level as compared to the normal group. The test compound, at low and high dose showed significantly decreased serum urea level as compared to the toxic control group. After treatment with standard ascorbic acid serum urea level was 31.64± 0.78 (Table 5; Fig. 5).

The study investigated the antioxidant activity of Plantago asiatica against Azathioprine-induced oxidative stress in rats. Results showed that Plantago asiatica effectively reduced oxidative damage parameters such as serum creatinine and serum urea in the kidneys, indicating its potential nephroprotective effects. Additionally, the study demonstrated an increase in superoxide...
Fig. 4: Effects of EEPA on serum creatinine levels in rats treated with azathioprine.

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

<table>
<thead>
<tr>
<th>Group name</th>
<th>Urea (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal group</td>
<td>26.4± 0.58</td>
</tr>
<tr>
<td>Toxic control (20 mg/kg)</td>
<td>56.8± 0.66</td>
</tr>
<tr>
<td>EEPA low dose (100 mg/kg)</td>
<td>47.9± 0.60**</td>
</tr>
<tr>
<td>EEPA high dose (200 mg/kg)</td>
<td>40.0± 0.41***</td>
</tr>
<tr>
<td>Standard ascorbic acid (10 mg/kg)</td>
<td>31.64± 0.78***</td>
</tr>
</tbody>
</table>

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

Fig. 5: Effects of EEPA on serum urea levels in rats treated with Azathioprine.
dismutase activity, further supporting the antioxidative properties of *Plantago asiatica*. The observed scavenging activity against DPPH free radicals agrees with previous research on the plant's antioxidant potential. Overall, the findings suggest that *Plantago asiatica* possesses promising antioxidant properties with potential therapeutic implications for oxidative stress-related disorders. Further research is needed to elucidate its mechanisms of action and broader therapeutic effects.

**Conclusion**

Based on findings of the present study, it may be suggested that *Plantago asiatica* has antioxidant activity against oxidative stress caused in rats by Azathioprine by reducing the levels of two oxidative stress indicators in the kidneys: serum urea and creatinine. *Plantago asiatica* has a higher antioxidant impact, according to assessments of antioxidant enzymes. In the renal tissues of rats exposed to azathioprine-induced oxidative stress, superoxide dismutase levels rise. *Plantago asiatica* demonstrated a high degree of scavenging activity against the DPPH system, which produces free radicals. *Plantago asiatica* possesses a wide range of pharmacological qualities, such as anthelmintic, anticancer, antimicrobial, analgesic, anti-inflammatory, anti-arthritic, and antibacterial characteristics.

**References**


