International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Issue 1 | JAN-MAR| 2018 | 658-664
Research Article | Pharmaceutical Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

EVALUATION FOR ANTI OXIDANT ACTIVITY OF *PLANTAGO ASIAATICA* EXTRACTS

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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 nephropretective 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 antihelmntic activities.

KEY WORDS

Plantago Asiatica, antioxidant activity.

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.[1] 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 [2]. 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.

Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids. Lipoprotein particles or membranes characteristically undergo the process of lipid peroxidation, giving rise to a variety of products including short chain aldehydes such as malondialdehyde or 4-hydroxynonenal, alkanes and alkenes, conjugated dienes and a variety of hydroxides and hydroperoxides [3-4]. Oxidative damage to proteins and nucleic acids similarly gives rise to a variety of specific damage products as a result of modifications of amino acids or nucleotides [5]. Such oxidative damage might also lead to cellular dysfunction and contribute to the pathophysiology of a wide variety of diseases.

Antioxidants serve as a defense against free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS form naturally during many metabolic processes, when regulated, they

contribute toward maintaining homeostasis in normal healthy cells and work as signaling molecules. However, the level of free radicals can increase if this balance is lost, which can happen in response to xenobiotics or environmental stress. When the balance is shifted towards pro-oxidants, a state of oxidative stress occurs, this condition can be a contributing factor to the development of several medical conditions, such as cardiovascular diseases, including atherosclerosis, various types of cancers, diabetes, and neurodegenerative diseases, like Parkinson's and Alzheimer's disease. Cells have several protective mechanisms against the harmful effects of ROS and RNS, both enzymatic (eg: SOD, CAT, GP_X) and non-enzymatic (eg: GSH, NADPH, α- tocopherol and ascorbic acid) [6-8].

The present study has been designed to achieve the following aims and objectives. To evaluate the antioxidant activity of *Plantago Asiaitica* 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.

Plantago species have been used since prehistoric times as herbal remedies. The herb is astringent,anti-toxic, antimicrobial, anti-inflammatory, anti-histamine,as well as demlcent, expectorant, styptic and diuretic. Externally, a poultice of the leaves is useful for insect bites, poison-ivy rashes, minor sores, and boils. In folklore it is even claimed to be able to cure snakebite. Internally, it is used for coughs and bronchitis, as a tea, tincture, or syrup. The broad-leaved varieties are sometimes used as a leaf vegetable for salads, green sauce, etc. Plantain seed husks expand and become mucilaginous when wet, especially those of P. psyllium, which is used in common over-the-counter bulk laxative and fiber supplement products such as Metamucil. P. psyllium seed is useful for constipation, irritable bowel syndrome, dietary fiber supplementation, and diverticular disease. Plantain has been consumed as human food since prehistory. For example, archaeological recovery along California's Central Coast has demonstrated use of this species as a food since the Millingstone Horizon.[9-10]

Psyllium supplements are typically used in powder form, along with adequate amounts of fluids. A dose of at least 7 grams daily taken with adequate amounts of fluid (water, juice) is used by some for management of elevated cholesterol. There are a number of psyllium products used for constipation. The usual dose is about 3.5 grams twice a day. Psyllium is also a component of several ready-to-eat cereals.[11]

Mucilage from Desert Indianwheat (Plantago ovata) is obtained by grinding off the husk. This mucilage, also known as Psyllium, is commonly sold as Isabgol, a laxative which is used to control irregular bowel syndrome and constipation. It is used as an indigenous Ayurvedic and Unani medicine for a whole range of bowel problems.[12] As Old English Wegbrade the plantago is one of the nine plants invoked in the pagan Anglo-Saxon Nine Herbs Charm, recorded in the 10th century. In Serbia, Romania, and Bulgaria, leaves from Plantago major are used as a folk remedy to preventing infection on cuts and scratches because of its antiseptic properties. In Slovenia and other Central European regions, the leaves were traditionally used topically as a cure for blisters resulting from friction (such as caused by tight shoes etc.) and as relief on mosquito bites in eastern Westphalia as well as western Eastphalia.[13]

MATERIALS:

The following are the chemicals or kits used in the present investigation.

Drugs and chemicals

Azathioprine is bought from RPG Life sciences Pvt, Ltd, Hyd. Ascorbic acid is bought from Finar chemicals, Ahmedabad, India. DPPH is bought from Sigma Aldrich, USA. Formaldehyde is bought from Finar chemicals, Ahmedabad, India. Normal saline is bought from Claris otsuka limited, Ahmedabad, India. Sodium citrate is bought from Finar chemicals, Ahmedabad, India.

Dipotassium hydrogen phosphate is bought from Merck Pvt, Ltd, Mumbai, India. Potassium dihydrogen phosphate is bought from Merck Pvt, Ltd, Mumbai, India. Diethyl ether is bought from Molychem, Mumbai, India. Chloroform is bought from Finar chemicals, Ahmedabad, India. O-dianisidine is bought from Sigma Aldrich, USA. Ethanol is bought from Merck Pvt, Ltd, Mumbai, India. Methanol is bought from Merck Pvt, Ltd, Mumbai, India. Riboflavin is bought from Sigma, st.louis, USA.

Kits used:

Urea is bought from Excel diagnostics Pvt, Ltd, Hyd, India. Creatinine is bought from Excel diagnostics Pvt, Ltd, Hyd, India.

METHODOLOGY

Collection and Authentification 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)

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.[14]

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 dissecator 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 = (weight of extract)/ (powder taken for extraction) × 100= 24.54/200 ×100 = 12.27 %.

***In Vitro* Method:**

DPPH scavenging activity procedure: - DPPH radical scavenging activity was measured using the method of Cotelle et al., with some modifications. 3 ml of reaction mixture containing 0.2 ml of DPPH (100 µM in methanol) 2.8 ml of test solution, at various concentrations (5, 10, 20, 40, 80, 160 320 µg/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:

% scavenging activity=absorbance of blank–absorbance of test / Absorbance of blank x 100+ 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 ± 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 were allowed *ad libitum.*

Acute toxicity study of *Plantago Asiaitica* 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 nontoxic 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^{o}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^{0}C used for the quantitative estimation of superoxide dismutase within 48hours by using uv spectrophotometer [UV method].[16-17]

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.

Normal value:

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

Estimation of Superoxide Dismutase (SOD)

0.88ml of riboflavin solution (1.3×10^{-5} M in 0.01M potassium phosphate buffer, pH 7.5) was added to 60µl of O-dianisidine solution (10^{-2} M in ethanol) and to this 100µl of clear separated SOD was added and optical density was measured at 460nm. Then the cuvette containing reaction mixture was transferred to the illuminating box, illuminated for 4min., and optical density was remeasured against blank containing ethanol in place of enzyme. The change in the optical density was determined. The SOD content was determined from the standard graph prepared using pure bovine SOD.

Estimation of serum Creatinine levels

To 1 ml of working reagent, 50µl 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 ΔA 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 = (ΔAT/ΔAS) *2.

Estimation of serum urea levels

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. [18-19]

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 = Absorbance test / Absorbance of standard x 40mg/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.

Statistical analysis:

All the values were expressed as mean ±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

RESULTS

***In Vitro* Evaluation of Antioxidant Activity of *Plantago Asiaitica* Dpph Radical Scavenging Activity:**

Table 2: Concentration dependent percentage inhibition of DPPH radical by various concentrations of test compound and ascorbic acid

Concentrations of test compound and ascorbic acid (µg/ml)	Percentage inhibition of DPPH radical (IC_{50})	
	***Plantago Asiaitica*(EEPA)**	**Ascorbic acid**
5	18.2±0.81	47.6±0.48
10	26.7±0.51	56.15±0.65
20	34.2±1.15	65.6±0.48
40	39.3±0.47	70±1.33
80	44.6±0.77	77.8±0.82
160	49.2±0.68	84.9±1.1
320	53.5±0.90	89.1±0.51

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 IC_{50} values were found to be as 18.2±0.81, to 53.5±0.9 increased with respectively concentrations with that of reference standard, ascorbic acid (47.6±0.48 to 89.1±0.51). 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.

***In Vivo* Studies**

Evaluation of Antioxidant Activity Using Azathioprine Induced Oxidative Stress In Rats

Superoxide Dismutase:

Superoxide dismutase is class of enzyme that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. It is an important antioxidant defense 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 were shown in below table, and figure.

Table 3: Superoxide dismutase levels in kidney tissue homogenate

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

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

In this study, we found that 20mg/kg dose of azathioprine 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 test compound at the dose of 100mg/kg and 200mg/kg after a 20mg/kg dose of azathioprine administration, shown a significant (p<0.001, p<0.0001) dose dependent increase in levels compared to toxic control group.

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 ±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± 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± 0.27mg/dl, has shown significance (***p<0.0001) and at high dose of test compound serum creatinine level was decreased that is 5.45± 0.15mg/dl, has shown significance (***p<0.0001). On treatment of standard ascorbic acid shows serum creatinine level 4.39± 0.11, has shown significance (***p<0.0001).

SERUM UREA:

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 ±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±0.62 mg/dl. The toxic control group shows serum urea level 58.7±0.60, increased compared to the normal group. The test compound, at low dose shows serum urea level 47.8±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±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±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 nephropretective 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 antihelmntic activities.

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