NTERNATIONAL JOURNAL OF PHARMACEUTICS & DRUG ANALYSIS
VOL.5 ISSUE 4, 2017; 96 – 107 ; http://ijpda.com; ISSN: 2348-8948

Review Article

ANTIOXIDANT EVALUATION OF *PHYLANTHUS AMARUS* AND *CASSIA AURICULATA* SEEDS FOR ITS HEPATOPROTECTIVE ACTIVITY

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Date Received: 27th March 2017; Date accepted: 5th April 2017; Date Published: 6th April 2017

Abstract

The hepatic and renal systems are highly prone to attack by generation of excessive concentration of free radicals. Many drugs, chemicals, pollutants may cause toxicity to these organ through mechanism mentioned above. Many antioxidants are of natural origin. Keeping the literature review and hypothesis of antioxidant principles and organ protection of selected medicinal plants like 70% methanolic extract of seeds of *Phyllanthus amarus* and *Cassia auriculata* has shown significant and dose dependant superoxide anion scavenging, hydroxyl radical scavenging, nitric oxide anion scavenging and reducing power. In addition, treatment with 70% methanolic extract 250 mg/kg seeds significantly restored the tissue GSH and reduced the lipid peroxidation. These results are indicating that the seeds also possess antioxidant property. Therefore the 70% methanolic extract 250 mg/kg seeds can be used as an organ protective potential against xenobiotics induced hepatotoxicity in rats.

The 70% methanolic extract of *Phyllanthus amarus* and *Cassia auriculata* seeds possess polyphenolic compounds like flavonoids, tannins and these polyphenolic compounds are known to possess antioxidant activity may be involved in organ protective activity. In the present study test extracts shown the presence of polyphenolic compound which function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors and synergists demonstrated antioxidant activity. Therefore, the antioxidant and organ protective property of 70% methanolic extract of *Phyllanthus amarus* and *Cassia auriculata* seeds was considered.

Keywords: *Phyllanthus amarus, Cassia auriculata,* Antioxidant & Hepatoprotective Activity

INTRODUCTION

The liver is vulnerable to a wide variety of metabolic, toxic, microbial, circulatory and neoplastic insults. Primary liver diseases are viral hepatitis, alcoholic liver disease and hepatocellular carcinoma. More often, hepatic damage is secondary, to some of the most common diseases in humans, such as cardiac decomposition, disseminated cancer and extrahepatic infections. The enormous functional reserve of the liver masks the clinical impact of early liver damage. However with progression of diffuse disease or strategic disruption of bile flow, the consequences of deranged liver function become life threatening.

Phyllanthus has a small pan-tropical herb common in gardens belonging to the family *Phyllanthraceae,* sub-family *Phyllanthoideae.* It grows 50 to 70 centimeters tall and bears ascending herbaceous branches. The bark is smooth and light green. It bears numerous pale green flowers which are often flushed with red. The fruits are tiny, smooth capsules containing seeds. The leaves are in one plane, stipules present, to 8*3.5 mm. flowers all pendent from one side of the branch, separate male and female. Fruit globular – depressed, splits into 3, seeds ribbed[1]. The species *Phyllanthus amarus* is commonly used as a diuretic and also indicated in the treatment of jaundice, hepatitis, kidney stones and diabetes. The leaves portion of the plant is used for itching and other skin infections, kidney stones, gonorrhea, diabetes and menstrual problems. The leaves portion of the plant is used for itching and other skin infections [2].

Figure. 1

Cassia auriculata L. belongs to the Fabaceae family and is used in traditional medicine, typically for skin disease, as a purgative, laxative, antihelminthic, antidiabetic and antioxidant from the ancient period. The pharmacological actions of *Cassia auriculata* L[3] as well as the antidiabetic and hypolipidemic efficacy of various parts (root, stem, leaves and flowers) of Cassia auriculata on alloxan-induced diabetic rats[4] have been documented. The present investigation was done to find whether the methanolic extract of *Cassia auriculata* leaf had hepatoprotective effect against carbon tetrachloride induced liver damage in Wister albino rats and to estimate the total antioxidant content, total phenolic content and total flavanoids of *C. auriculata* methanolic leaf extract.

The total protein level and lipid peroxidation of MDA production were significantly decreased in drug treated group than the induced rat group. This biochemical changes indicate in liver shows recovery of damaged liver by *C. auriculata* dried leaf extract. The non-antioxidant vitamin E and reduced glutathione shows in elevated level in post treated rat group while comparing with CCl_4 induced group and nearest to control group. The enzymatic antioxidant enzymes superoxide dismutase, glutathione-s-transferase, glutathione peroxidase and catalase level was significantly increased level in post treated rat group than the carbon tetra chloride induced group[5].

MATERIALS AND METHODS:

Plant material

The seeds of *Phyllanthus amarus* and *Cassia auriculata* were collected from local gardens of Tirupati. The plant was identified and authenticated by Madhava Chetty, Department of Botany, Sri Venkateshwara University, Tirupati, Andhra Pradesh. A herbarium specimen is deposited in Nizam Institute of Pharmacy and Research Centre, Hyderabad, India.

Preparation of extracts

The crushed and dried seeds of *Phyllanthus amarus* and *Cassia auriculata* were divided into two parts; one part was extracted successively with petroleum ether, benzene, chloroform and finally with

methanol by soxhlet extraction and concentrated by rotary vacuum[6]. The other part is extracted by cold maceration process for aqueous extraction[4]. The obtained extracts were dried by evaporation. The yield 18.05% w/w and 15.32% w/w were stored in refrigerator and weighed quantities were suspended in tween 80 and 2% tragacanth solution respectively, for the experiment. The extracts were used for In-vitro and In-vivo antioxidant studies. The samples were dried at room temperature, subsequently milled into powder and stored in air-tight stopped glassware before subject to preliminary qualitative phytochemical analysis.

The extracts were concentrated under reduced pressure and stored in a desicator until further use and the percentage yield of corresponding extracts were calculated.

Experimental animals

Healthy adult Wister rats, weighing 180-200g of either sex were used in this study. They were procured from Mahaveer agencies, Hyderabad. The animals were allowed to acclimatize for 7 days under laboratory conditions, before the experiment[7]. They were housed in polypropylene cages and maintained at 25˚C ± 2˚C under 12 hours dark / light cycle. The rats had free access to standard pellet chow (Gold Mohar Lipton India Ltd.) and water *ad libitum* throughout the experiment. They were housed in a cage of six animals per cage and were renewed thrice a week to ensure hygienity and maximum comfort for animals. The well maintained Nizam Institute of Pharmacy & Research Centre animal house held at Deshmukhi, Hyderabad was approved by CPCSEA bearing Reg. No. 1330/ac/10/CPCSEA. Ethical clearance for handling the animals was obtained from the Institutional Animals Ethical Committee (IAEC) bearing Lr. No. NIPRC/IAEC/PhD/2014/07. Prior to the beginning of the project work.

STANDARD DRUG *(Silymarin 35mg /5ml):*

Hepatoprotection

Silymarin, an Ayurvedic formulation of Micro drug company, Bangalore is used mainly in the treatment of liver disorders. Silymarin was administered in a dose of 100 mg/kg p.o., into rats. **Methods for hepatoprotective evaluation**

A. <u>In-vitro antioxidant activity:</u>

The following in-vitro models were carried out to evaluate antioxidant activity of *Phyllanthus amarus* and *Cassia auriculata* seeds.

1. Reducing power.
2. Superoxide anion scavenging activity.
3. Hydroxyl radical scavenging activity.
4. Nitric oxide radical scavenging activity.

1. *Reducing power*

The reducing power of methonolic and aqueous extracts of *Phyllanthus amarus* seeds were determined according to the method of Oyaizu[8].

Procedure:

Extracts of *Phyllanthus amarus* and *Cassia auriculata* seeds were mixed in 1ml of distilled water so as to get 20 µg, 40 µg concentrations. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50ºC for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance (OD) was measured at 700 nm.

Increased absorbance of the reaction mixture indicates increased in reducing power.

The percent reducing power was calculated by using the formula:

$$\% \text{ increase in absorbance} = \frac{\text{Control OD-Test OD}}{\text{Control OD}} \times 100$$

The results are complied in the Table No. 1 and graphically shown in Fig. No. 2

2. *Superoxide anion scavenging activity*

Oxygen is essential for the survival of aerobic cells, but it has long been known to be toxic to them when supplied at concentration greater than those in normal air. The biochemical mechanisms responsible for oxygen toxicity include lipid peroxidation and the generation of $H_2O_2^+$ the superoxide radical, O_2^+. This superoxide radical can inhibit or propagate the process of lipid peroxidation. Hence the superoxide anion scavenging activity of *Phyllanthus amarus* and *Cassia auriculata* seeds were measured[9].

Procedure:
About 1ml of nitro blue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), and 0.1 ml of sample solution of methanolic extract of *Phyllanthus amarus, Cassia auriculata* seeds and standard in water was mixed. The reaction was started by adding 100 μl of phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 minutes, and the absorbance at 560 nm was measured against blank.

Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. % inhibition of OD was calculated by using the formula mentioned earlier.

3. *Hydroxyl radical scavenging activity*
In the biochemical system, superoxide and H_2O_2 react to form the hydroxyl radical which can attack and destroy almost all known biochemicals[10]. Phenylhydrazine when added to erythrocyte hosts cause peroxidation of endogenous lipids and alteration of membrane fluidity. The damage of erythrocytes is probably initiated by active oxygen species like $O_2^{\bullet}$, $OH^{\bullet}$ and H_2O_2 which are generated in solution from auto-oxidation of phenyl hydrazine. This forms the basis of this experiment.

Procedure: Hydroxyl radical generation by phenyl hydrazine has been measured by the 2-deoxyribose degradation, assay of Halliwell and Gutteridge[11]. In 50 mM phosphate buffer (pH 7.4), 1 mM deoxyribose and 0.4 ml of methanolic extract of *Phyllanthus amarus, Cassia auriculata* seeds and standard were taken. 0.2 ml phosphate buffer was added to make reaction solution 1.6 ml after 10 min incubation 0.4 ml of 0.2 mM phenyl hydrazine was added. Incubation was terminated after 1hr and 4 hrs and 1ml each of 2.8% TCA and 1% (w/v) thiobarbituric acid were added to the reaction mixture and heated for 10 minutes in a boiling water bath. The tubes were cooled and absorbance was taken at 532 nm.
Decreased in absorbance is indicating the hydroxyl free radical scavenging activity. The % reduction in the OD is calculated.

4. *Nitric oxide radical scavenging activity*
Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons etc. involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which acts as free radicals. This forms the basis of this experiment[12].

Procedure:
Nitric oxide (NO) radial were generated from sodium nitroprusside solution of physiological pH. Sodium nitroprusside (1 ml of 10 mM) were mixed with 1ml of methonolic extract of *Phyllanthus amarus* and *Cassia auriculata* seeds of different concentration (20-100 μg/ml) in phosphate buffer (pH 7.4). The mixture is incubated at 25ºC for 150 min. To 1ml of incubated solution, 1 ml Griess's reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546nm. Percentage inhibition of OD was calculated by using the earlier formula.
The results are complied in the Table No. 4 and graphically shown in Fig. No. 5

RESULTS:
Reducing power activity of extracts of *P. amarus* & *C. auriculata* seeds:
It was observed that 70% methanolic extract demonstrated dose dependent increase in the reducing property. 25 mcg sodium metabisulphate (Standard) showed 73.09% reducing property. 70% methanolic extract at 40 mcg had all most same reducing property as 25 mcg sodium metabisulphate i.e. 70.51%
It was observed that 70% methanolic extract demonstrated dose dependent percentage increase in superoxide anion scavenging activity. 25mcg sodium metabisulphate (Std) had 73.89% scavenging activity. 70% methanolic extract showed lesser activity than standard i.e. 55.59%.

Hydroxyl radical scavenging activity of extracts of *P. amarus* & C. ariculata seeds:
It was observed that 70% ethanolic extract demonstrated dose dependent percentage increase in the hydroxyl radical scavenging activity. 25 mcg sodium metabisulphate (Standard) showed 56.47% (for 1hour incubation period) and 55.06% (for

4hours incubation period). 70% methanolic extract showed lesser activity in case of 1 hour incubation period i.e. 65.90%, but showed higher in case of 4hours incubation period i.e. 78.27%.

Table 1: Reducing power activity of extracts of *P. amarus* & *C. auriculata* Seeds

Groups	Absorbance Mean ± SEM	% Increase
Control	0.371 ± 0.001	--
Control + Standard 25 µg	0.296 ± 0.002***	73.09
P. amarus meth. extract 20 µg	0.182 ± 0.001*	53.43
P. amarus meth. extract 40 µg	0.216 ± 0.002***	60.31
C. auriculata meth. extract 20 µg	0.269 ± 0.002***	65.47
C. auriculata meth. extract 40 µg	0.288 ± 0.002***	70.51

Values are the mean ± S.E.M., n=3; Significance ***P<0.001 and *P<0.05 compared to control; Std: Sodium metabisulphide

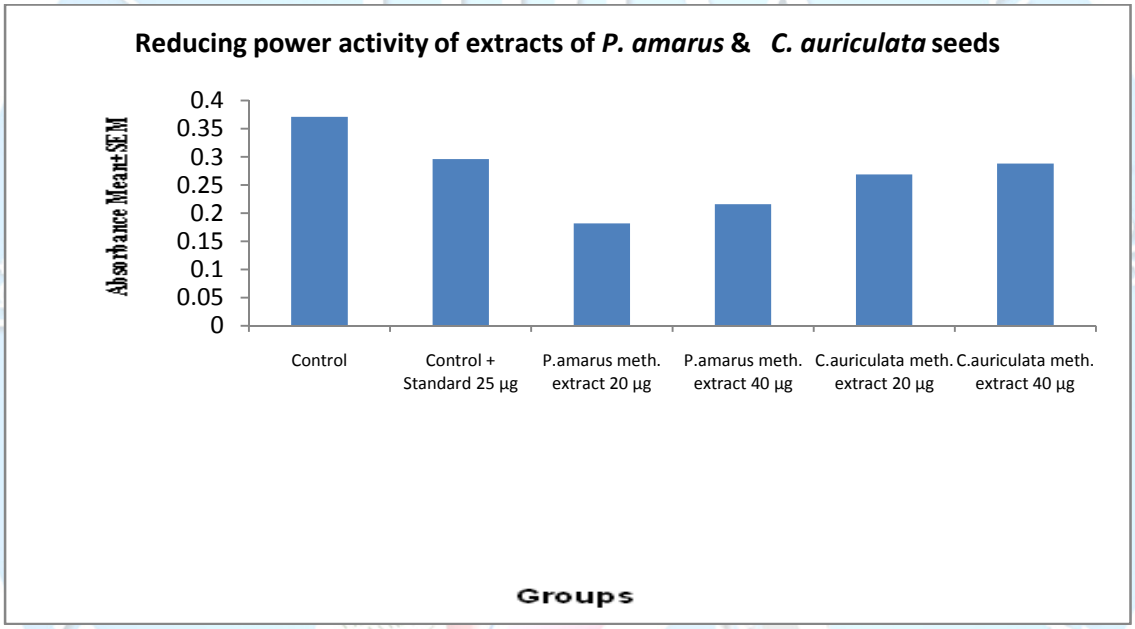


Figure. 2: Superoxide anion scavenging activity of extracts of *P. amarus* & *C. ariculata* seeds

Nitric oxide radical scavenging activity of methanolic extracts of *P. amarus C. auriculata* seeds:
It was observed that 70% methanolic extract demonstrated dose dependent percentage inhibition in the nitric oxide radical scavenging activity. 25 mcg sodium metabisulphate (Standard) showed 70.52% activity. 70% methanolic extract of P. amarus had less nitric oxide radical scavenging activity at 40 mcg compared to 25 mcg sodium metabisulphate i.e. 65.08%.

B. Invivo Antioxidant Activity

The extracts of *Phyllanthus amarus* and *Cassia auriculata* seeds on Glutathione (GSH) and Lipid peroxidation estimation in thioacetamide induced hepatotoxicity in rats.

1. Glutathione estimation

Tissue samples will be homogenized in ice cold Trichloroacetic acid (1gm tissue + 10ml 10% TCA) in an ultra turrax tissue homogenizer. Glutathione measurements will be performe using a modification of the Ellamn procedure, after centrifugation at 3000 rpm for 10 minutes, 0.5ml supernatant will be added to 2ml of 0.3 M disodium hydrogen phosphate solution. A 0.2ml solution of dithiobisnitrobenzoate (0.4 mg/ml in 1% sodium citrate) will be added and the absorbance at 412 nm measure

immediately after mixing. Percentage increase in OD is directly proportional to the increase in the GSH. Hence percentage increase in OD is calculated [23].

Table. 2
Superoxide anion scavenging activity of extracts of *P. amarus* & *C. auriculata* seeds

Group	Absorbance Mean±SEM	% Increase
Control	0.862±0.020	---
Control + Std 25µg	0.525±0.001***	73.89
P. amarus meth. extract 20 µg	0.469±0.001***	55.59
P. amarus meth. extract 40 µg	0.422±0.002***	51.04
C. auriculata meth. extract 20 µg	0.279±0.002**	46.25
C. auriculata meth. extract 40 µg	0.326±0.002**	48.50

Values are the mean ± S.E.M., n=3
Significance ***P<0.001 compared to control Sig. **P<0.01
Std: Sodium metabisulphate

Figure. 3: Hydroxyl radical scavenging activity of extracts of *P. amarus* & C. ariculata seeds

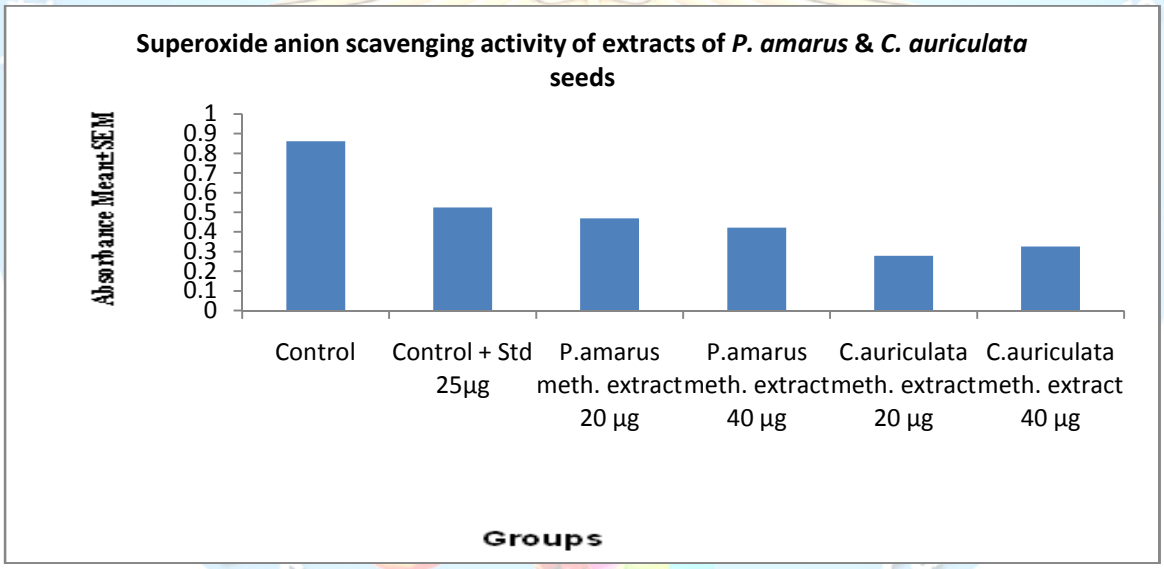
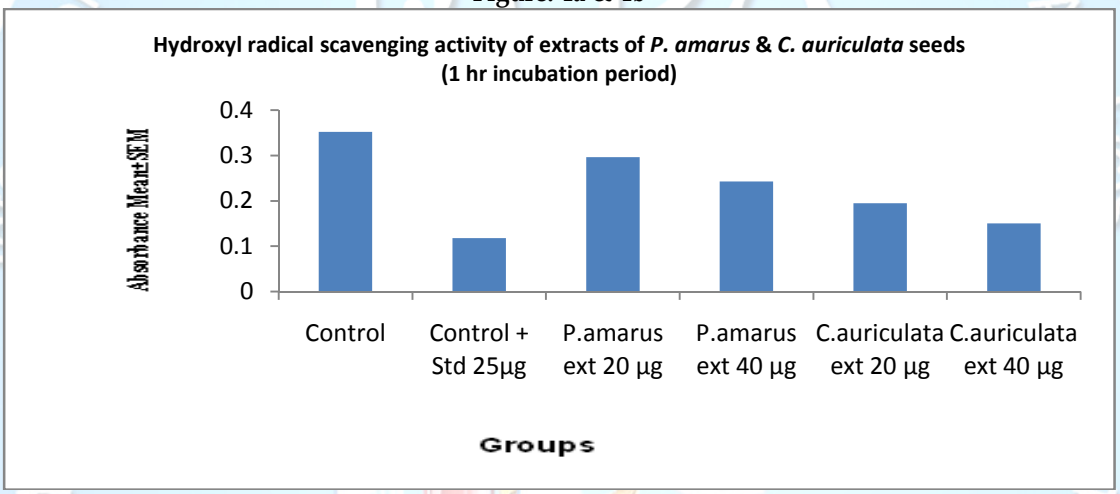


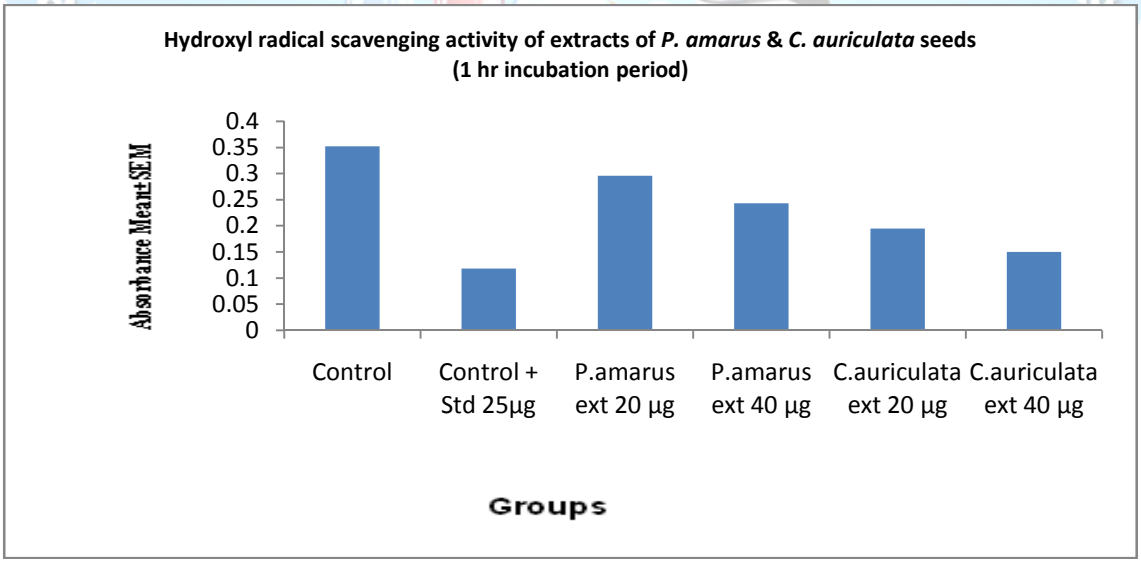
Table. 3: Hydroxyl radical scavenging activity of extracts of *P. amarus* & *C. auriculata* seeds

Group	Absorbance Mean±SEM	% Inhibition	Absorbance Mean±SEM	% Inhibition
Control	0.352±0.0005	---	0.405±0.0020	---
Control + Std 25µg	0.118±0.0037**	56.47	0.182±0.0040*	55.06
P. amarus meth. extract 20 µg	0.296±0.0028***	65.90	0.331±0.003***	78.27
P. amarus meth. extract 40 µg	0.243±0.0020***	60.96	0.253±0.001**	68.14
C. auriculata meth. extract 20 µg	0.195±0.0030*	40.58	0.206±0.002**	58.55
C. auriculata meth. extract 40 µg	0.150±0.0035*	52.10	0.189±0.003*	55.14

Values are the mean ± S.E.M., n=3, *P<0.05 compared to control Significance, **P<0.01 compared to control, ***P<0.001 compared to control. Std : Sodium metabisulphate

Figure. 4a & 4b





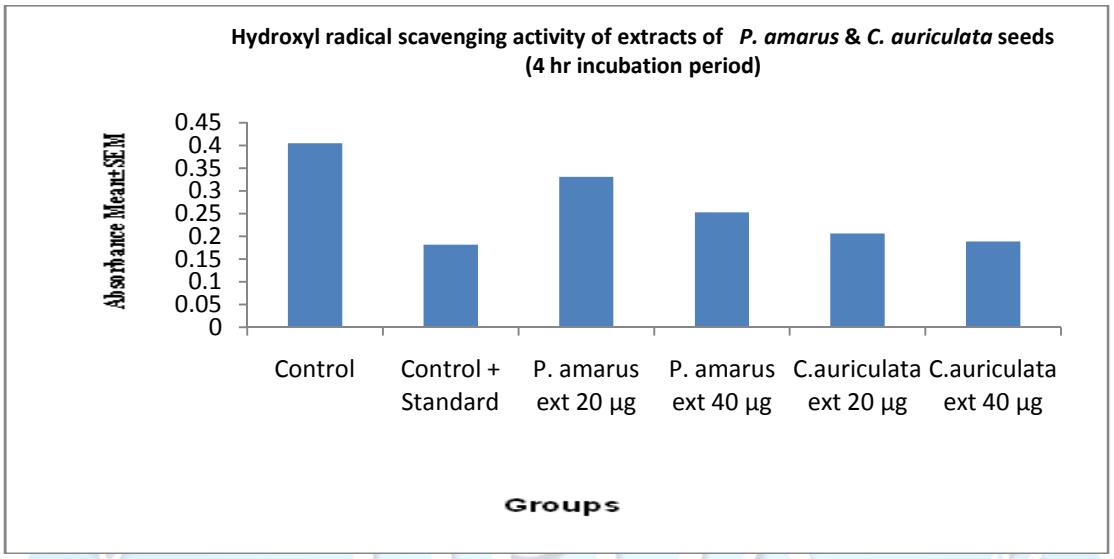


Table. 4: Nitric oxide radical scavenging activity of extracts of *P. amarus* & *C. auriculata* seeds

Group	Absorbance Mean±SEM	% Increase
Control	0.397±0.0015	---
Control + Std 25µg	0.247±0.0015***	70.52
P. amarus meth. extract 20 µg	0.212±0.0008**	64.04
P. amarus meth. extract 40 µg	0.218±0.0017***	65.08
C. auriculata meth. extract 20 µg	0.174±0.0017**	59.13
C. auriculata meth. extract 40 µg	0.112±0.0014**	53.25

Values are the mean ± S.E.M., n=3 Significance***P<0.001 compared to control; Std: Sodium metabisulphate

Figure. 5

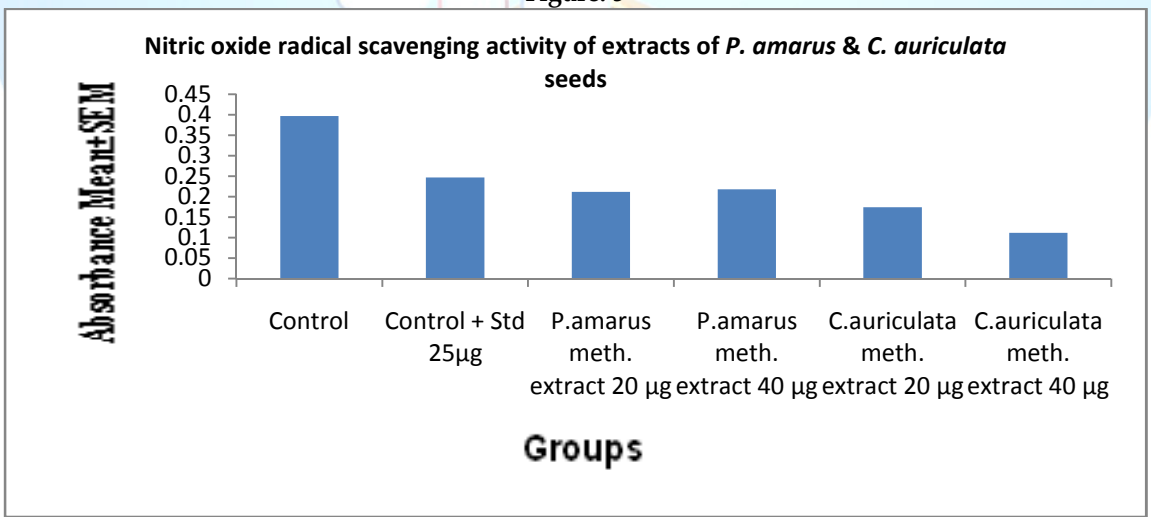


Figure. 5

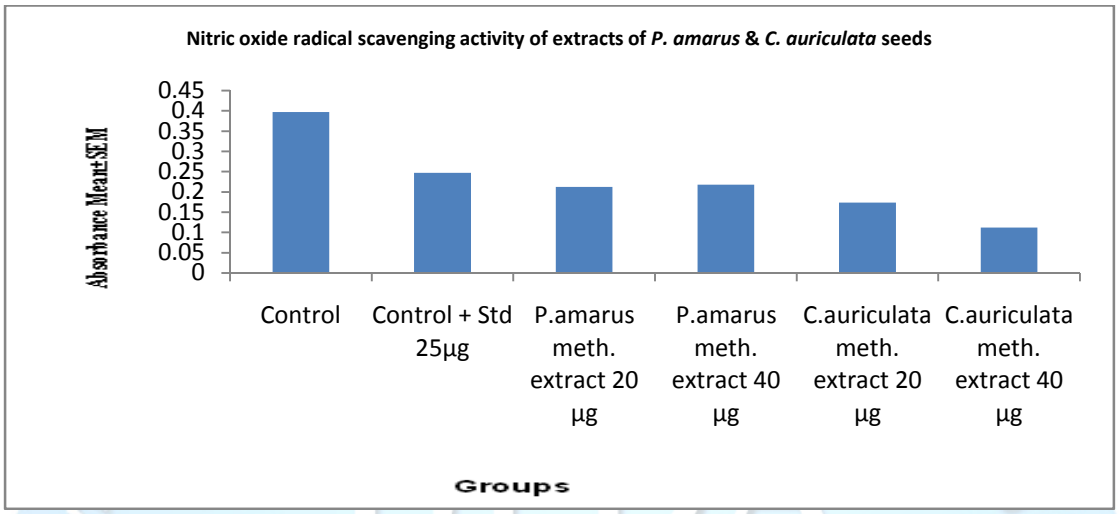


Table. 5: **Effect of meth. extracts of *P. amarus* & *C. ariculata* seeds on GSH level in Thioacetamide induced hepatotoxicity**

Treatment	Absorbance Mean ± SEM	% Increase
Negative control (1ml vehicle)	0.921 ± 0.007	--
Positive control Thioacetamide + Tween 80 (1:1) 100 mg/kg, s.c	0.506 ± 0.010	--
Thioacetamide + Std (Silymarin) 100 mg/kg, s.c + 100 mg/kg, p.o	0.898 ± 0.009***	77.47
Thioacetamide + P. amarus ext. 100 mg/kg, s.c + 150 mg/kg, p.o	0.808 ± 0.008**	60.68
Thioacetamide + P. amarus ext. 100 mg/kg, s.c + 250 mg/kg, p.o	0.891 ± 0.009***	76.08
Thioacetamide +C. auriculata ext. 100 mg/kg, s.c + 150 mg/kg, p.o	0.709 ± 0.011*	50.68
Thioacetamide +C. auriculata ext. 100 mg/kg, s.c + 250 mg/kg, p.o	0.795 ± 0.008***	56.08

Values are the mean ± SEM of six rats / treatment; Significance ***P<0.001, **P<0.01, *P<0.05 compared to thioacetamide treatment

Figure. 6

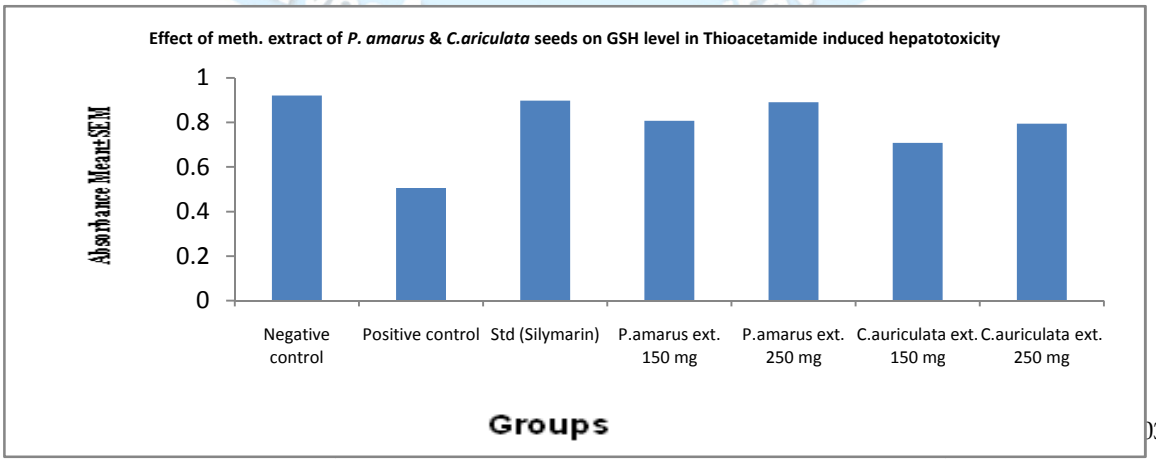
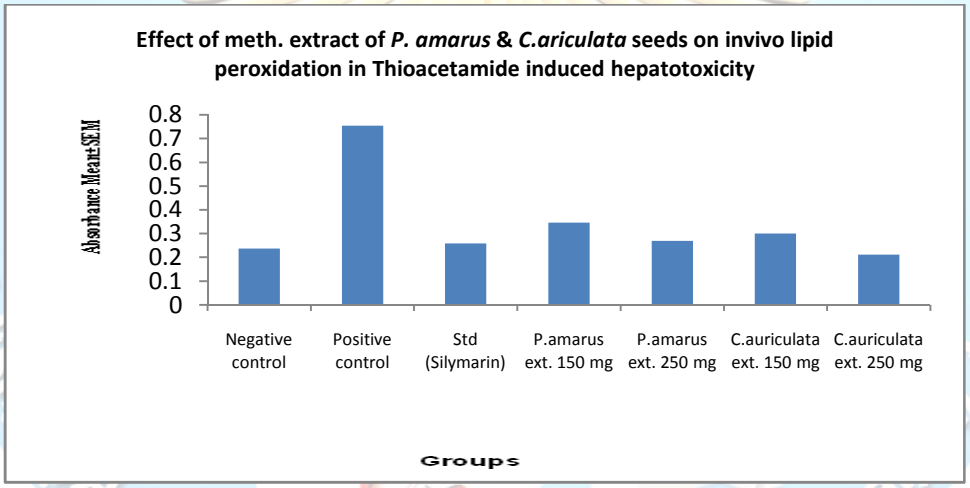


Table. 6: Effect of meth. extract of *P. amarus* & *C. ariculata* seeds on invivo lipid peroxidation in Thioacetamide induced hepatotoxicity

Treatment	Absorbance Mean ± SEM	% Inhibition
Negative control (1ml vehicle)	0.237 ± 0.008	--
Positive control 100 mg/kg, s.c Thioacetamide + Tween 80 (1:1)	0.754 ± 0.008	--
Thioacetamide + Std (Silymarin) 100 mg/kg, s.c + 100 mg/kg, p.o	0.259 ± 0.003***	65.91
Thioacetamide + P. amarus ext. 100 mg/kg, s.c + 150 mg/kg, p.o	0.346 ± 0.013**	43.64
Thioacetamide + P. amarus ext. 100 mg/kg, s.c + 250 mg/kg, p.o	0.270 ± 0.007***	64.19
Thioacetamide +C. auriculata ext. 100 mg/kg, s.c + 150 mg/kg, p.o	0.300 ± 0.015**	40.47
Thioacetamide +C. auriculata ext. 100 mg/kg, s.c + 250 mg/kg, p.o	0.211 ± 0.009***	59.16

Values are the mean ± SEM of six rats / treatment; Significance ***P<0.001, **P<0.01 compared to thioacetamide treatment

Figure. 7



2. Lipid peroxide estimation

The degree of lipid peroxide formation is assaye by monitoring thiobarbituric reactive substance formation. Stock solution of TCA-TBA-HCl reagent: 15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid. This solution may be mildly heated to assist in the dissolution of the thiobarbituric acid [24]. Combine 1ml of biological sample (o.1-2.0mg of membrane protein or 0.1-0.2µmol of lipid phosphate) with 2ml of TCA-TBA-HCl and mix thoroughly. The solution is heated for 15 minutes in a boiling water bath. After cooling, the flocculent precipitate will be removed by centrifugation at 1000 rpm for 10 minutes. The absorbance of the sample is determined at 535 nm against a blank that contains all the reagents minus the lipid. The malondialdehyde concentration of the sample can be calculated by using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Influence of meth. extracts of *P. amarus* & *C. ariculata* seeds on invivo GSH level in Thioaceta-

mide induced hepatotoxicity:

There was a marked depletion of GSH level in Thioacetamide treated groups. 100 mg/kg silymarin has increased it by 77.47%. 70% methanolic extract showed a dose dependent increase in the levels of GSH. However at 250 mg/kg 70% methanolic extract showed 76.08% which was almost near to standard silymarin.

Influence of 70% methanolic extract of *Phyllanthus amarus* and *C. auriculata* seeds on invivo lipid peroxidation in Thioacetamide induced hepatotoxicity:

There was dose dependent inhibition of in vivo lipid peroxidation by both the doses of 70% methanolic extract. 100mg/kg silymarin showed 65.91% inhibition, whereas 250 mg/kg 70% methanolic extract showed 64.19% inhibition, which was almost near to standard silymarin.

DISCUSSION AND CONCLUTION:

Many toxins, xenobiotics, environmental pollutants are exposed to stressful conditions which enhances the body metabolic rate resulting in the production of several free radicals. These free radicals bind covalently with the tissue macromolecules leading to the cell necrosis. However, there are inbuilt antioxidant systems like tissue GSH, superoxide desmustase, catalase, *etc*. The excessive generation of free radicals results in the cell necrosis. Since liver and kidney are prone to the attack by the free radicals leading to cell necrosis. Therefore antioxidants are normally administered to treat or prevent the organic toxicities.

The 70% methanolic extract of seeds of *Phyllanthus amarus* and *Cassia auriculata* has shown significant and dose dependant superoxide anion scavenging, hydroxyl radical scavenging, nitric oxide anion scavenging and reducing power. In addition, treatment with 70% methanolic extract 250 mg/kg seeds significantly restored the tissue GSH and reduced the lipid peroxidation. These results are indicating that the seeds also possess antioxidant property. Therefore the 70% methanolic extract 250 mg/kg seeds can be used as an organ protective potential against xenobiotics induced hepatotoxicity in rats.

The 70% methanolic extract of *Phyllanthus amarus* and *Cassia auriculata* seeds possess polyphenolic compounds like flavonoids, tannins and these polyphenolic compounds are known to possess antioxidant activity may be involved in organ protective activity. In the present study test extract shown presence of polyphenolic compound and demonstrated antioxidant activity. Therefore, the antioxidant and organ protective property of 70% methanolic extract of *Phyllanthus amarus* and *Cassia auriculata* seeds was considered.

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