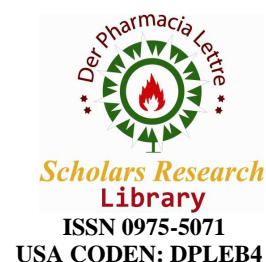




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Evaluation of *In-vitro* antioxidant methods of *cassia auriculata*

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ABSTRACT

Antioxidant studies were carried out for methanolic extract of *Cassia auriculata* seeds for proving its utility in inflammation and healing mechanism. The methanolic extract was screened for antioxidant activity by nitric oxide radical scavenging, lipid peroxidation inhibition and DPPH methods at different concentrations. Throughout the studies seeds extract showed marked antioxidant activity. The antioxidant activity of the seeds extract may be due to stabilization of plasma membrane, thereby lowering the elevated levels of serum lysosomal enzymes. The antioxidant activity was found to be concentration dependent and may be attributed to the presence of high flavanoids and bioflavonoids content in the seeds of *Cassia auriculata*.

Keywords: Free radicals, 1, 1- diphenyl-2-picrylhydrazyl, Spectrophotometer, *In-vitro*, nitric oxide.

INTRODUCTION

Oxidative stress has been associated with the pathogenesis of many human diseases; the use of antioxidants in therapeutics is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. Antioxidants are widely used as ingredients in dietary supplements maintaining health and preventing diseases such as cancer and coronary heart disease [1].

Cassia auriculata, Linn (Caesalpiniaceae) commonly known as Tanners *Cassia* [Avaram] is a shrub with large bright yellow flowers, growing wild in Central Provinces and Western peninsula parts of India [2]. It is one of the main constituents of 'Kalpa herbal tea' and has proven antidiabetic action [3]. The five parts of the plant roots, bark, leaves, flowers, and unripe fruits taken in equal quantity, dried and powdered known as 'Avarai panchaga choornam' has beneficial effect in diabetes [4]. The flowers are used in urinary discharges, nocturnal emissions, diabetes, throat troubles. The roots are alexeteric, useful in urinary discharges, tumors, skin diseases, asthma. The leaves are used as anthelmintic, in ulcers, leprosy, skin

diseases [5]. The plant has been reported to possess hepatoprotective [6], antiperoxidative and antihyperglycemic activity [7], and microbicidal activity [8].

Since antioxidant and membrane stabilizing activity have not been systematically studied in the plant parts, therefore *in vitro* antioxidant studies were undertaken which could be the major mechanism involved in the protective effect.

MATERIALS AND METHODS

Collection, authentication and extraction:

Fresh seeds of *Cassia auriculata* were collected from TamilNadu, India in August-September and authenticated at Agharkar Research Institute, Pune. The plant part was dried in an oven below 60°C for 2 hrs. The dried part of plant was finely powdered and extracted with 80% aqueous methanol using Soxhlet apparatus at 55°C. The soluble part was concentrated over water bath maintained below 60°C and dried in a vacuum oven to obtain free flowing reddish brown powder.

Experimental animals:

Albino mice of Swiss strain (20-25kg) were purchased from Bharat Serum and Vaccines, Thane. The animals were housed in polypropylene cages and maintained under standard conditions (12 hours light/12 hours dark cycle; $25 \pm 3^\circ\text{C}$; humidity 35-60 %). They were fed with Amrut brand pelleted standard diet manufactured by Nav Maharashtra Chakan oils, Ltd., Maharashtra and drinking water *ad libitum*. The animals had free access to water all the time and were allowed to adapt to the animal house conditions by keeping them for a period of 8-10 days prior to using them for the experiments. The study was conducted after seeking clearance from the Institutional animal ethical committee.

Chemicals and reagents

Acetic acid, Ascorbic acid, Ferrous sulphate, Potassium chloride, Sodium bicarbonate, Sodium carboxy methyl cellulose, Sodium hydroxide, Sodium lauryl sulphate, Sodium nitroprusside, Sulphuric acid and Tris-HCl buffer were procured from Sd Fine Chem Ltd, Mumbai.

Glacial Acetic Acid, n-Butanol, Pyridine were obtained from Sisco Labs., Mumbai.

Sodium Chloride, Ethanol were procured from Merck Ltd., Mumbai.

1, 1-Diphenyl-2-picrylhydrazyl (DPPH), Sulphanilamide, Phosphoric acid, 0.1% naphthylethylenediamine dihydrochloride from Hi-Media, Mumbai.

Instruments

UV-Visible Spectrophotometer (Shimadazu) for carrying antioxidant studies

Homogenizer to homogenize liver for in-vitro lipid peroxidation studies.

Evaluation of antioxidant activity

Nitric oxide scavenging activity

1.5 ml, 10 mM sodium nitroprusside in phosphate buffer saline pH 7.4 was mixed with 0.5ml various concentrations (5mM to 50mM) of MECA and the mixture was incubated at 25° C for 150 min. During which sodium nitroprusside spontaneously generates nitric oxide. After the incubation 1.5 ml Griess reagent (1% Sulphanilamide, 2% Phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride) was added. The reaction mixture incubated at room temperature for 30 min. The absorbance was measured with UV-spectrophotometer at 540 nm [9].

The activity of MECA was compared with ascorbic acid which was used as a standard antioxidant. The nitric oxide scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = \frac{(A_0 - A_1)}{(A_0)} \times 100$$

Where

A_0 is the absorbance of the control (blank, without MECA)

A_1 is the absorbance in the presence of the MECA or the standard ascorbic acid.

In vitro lipid peroxidation

Preparation of liver homogenate:

The liver that was excised from the mice was weighed and chilled in ice cold saline. After washing with ice cold saline tissue homogenates was prepared in a ratio of 1g of wet tissue to 9ml of KCl.

Assay Procedure:

The reaction mixture contained 0.2 ml, 10% w/v mice liver homogenate in 0.2 ml, 0.15 M Potassium Chloride, 0.4 ml Tris buffer pH 7.5 and various concentrations (5-100 $\mu\text{g/ml}$) of MECA. Lipid peroxidation was initiated by addition of 0.1 ml, 10 μM Ferrous Sulphate and 0.1 ml, 100 μM Ascorbic Acid. The reaction mixture was incubated at 37° C for 1h. After the incubation, reaction was terminated by adding 2 ml 0.8% w/v thiobarbituric acid. The contents were heated at 95° C for 15 min for development of colored complex. The tubes were cooled and centrifuged at 4000 rpm for 10 min and supernatant were removed and its color intensity was measured at 532 nm [10]. The activity of MECA was compared with curcumin which was used as a standard in lipid peroxidation. The inhibition of lipid peroxidation was calculated according to the following equation:

$$\% \text{ inhibition} = \frac{(A_0 - A_1)}{(A_0)} \times 100$$

Where

A_0 is the absorbance of the control (blank, without MECA)

A_1 is the absorbance in the presence of the MECA or standard curcumin.

DPPH Radical method:

The reaction mixture consisted of 1 ml of 0.1mM DPPH in ethanol, 0.95 ml of 0.05 M Tris-HCl buffer (pH 7.4), 1 ml of ethanol and 0.05 ml of MECA at various concentrations (10mg/ml to 100mg/ml). The absorbance of the mixture was measured at 517 nm exactly 30 sec after adding MECA. The % of scavenging activity was determined by comparing the result of MECA with those of standard antioxidant ascorbic acid [11].

$$\% \text{ of scavenging activity} = 100 - (100 / \text{blank absorbance} \times \text{Standard} / \text{sample absorbance})$$

Where

Blank absorbance is the absorbance that is without the MECA.

Sample absorbance is the absorbance of MECA.

Statistical analysis

The data was analyzed using SPSS packages (version 6.0) and IC_{50} values representing the concentration required to induce 50 % inhibition was determined from the linear segment of the curve obtained by plotting % inhibition on Y axis versus concentration of MECA concentration on X axis of flowers. The MECA was compared with standard antioxidant drug having IC_{50} and the correlation coefficient (R^2) were calculated from the graph.

RESULTS*Evaluation of antioxidant activity**Nitric oxide scavenging activity*

MECA seeds showed a significant free radical scavenging action against nitric oxide (NO) induced release of free radicals. The IC_{50} values of standard drug and plant part were found to be 22.74mM and 43.38 mM whereas R^2 was found to be 0.9988 and 0.9964 (Table no.1 and 2, Figure no.1 and 2).

In vitro lipid peroxidation

MECA seeds elicited concentration dependent inhibition of $FeSO_4$ induced lipid peroxidation in mice liver homogenate. The IC_{50} values of standard drug and plant part were found to be 30.67 μ g/ml, 43.99 μ g/ml whereas R^2 was found to be 0.9979 and 0.9955. (Table no.3 and 4, Figure no.3 and 4).

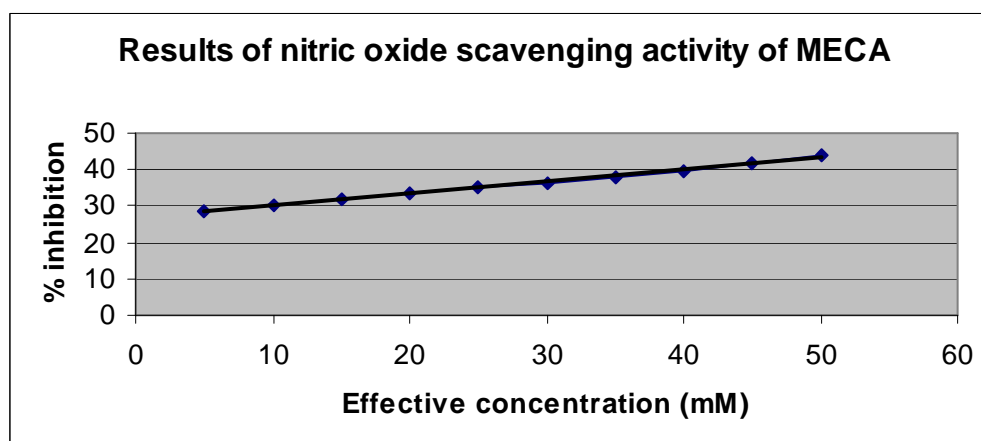
Table 1: Nitric oxide radical scavenging activity of MECA

Effective concentration (mM)	% of nitric oxide scavenging activity	IC_{50} value
5	28.69	43.38 mM
10	30.08	
15	31.69	
20	33.33	
25	35.03	
30	36.41	
35	38.03	
40	39.70	
45	41.73	
50	44.00	

Table 2: Nitric oxide radical scavenging activity of Ascorbic acid

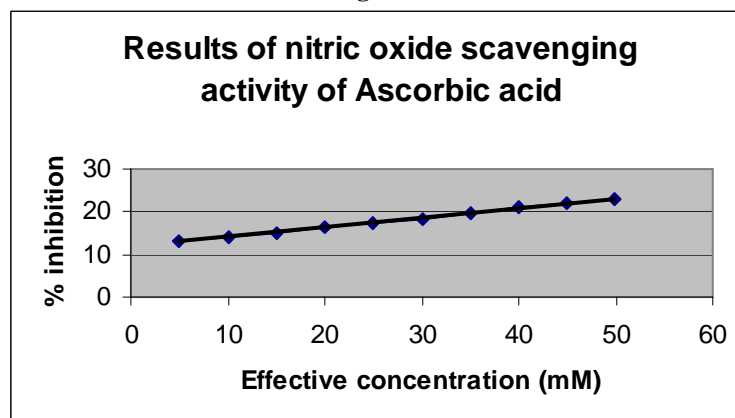
Effective concentration (mM)	% of nitric oxide scavenging activity	IC_{50} value
5	13.11	22.74mM
10	14.05	
15	15.18	
20	16.25	
25	17.20	
30	18.43	
35	19.59	
40	20.97	
45	22.05	
50	23.10	

Figure 1



Equation for the line for the linear segment of the curve
 $Y=0.3332x + 26.707$ and $R^2= 0.9964$
 $IC_{50} = 43.38$ mM

Figure 2



Equation for the line for the linear segment of the curve
 $Y=0.2159x + 11.941$ and $R^2 = 0.9988$
 $IC_{50} = 22.74$ mM.

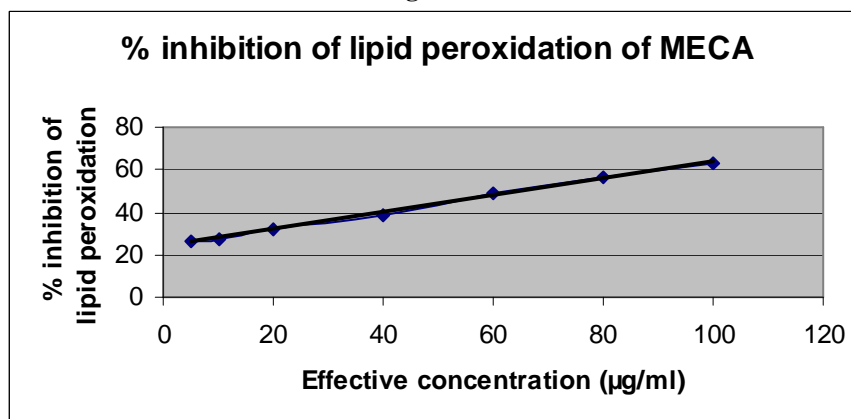
Table 3: Lipid Peroxidation Inhibitory Activity of MECA

Effective concentration ($\mu\text{g/ml}$)	% Inhibition in TBARs formation	IC_{50} value
5	26.44	43.99 $\mu\text{g/ml}$
10	27.75	
20	32.42	
40	38.70	
60	49.27	
80	56.71	
100	62.77	

DPPH Radical method

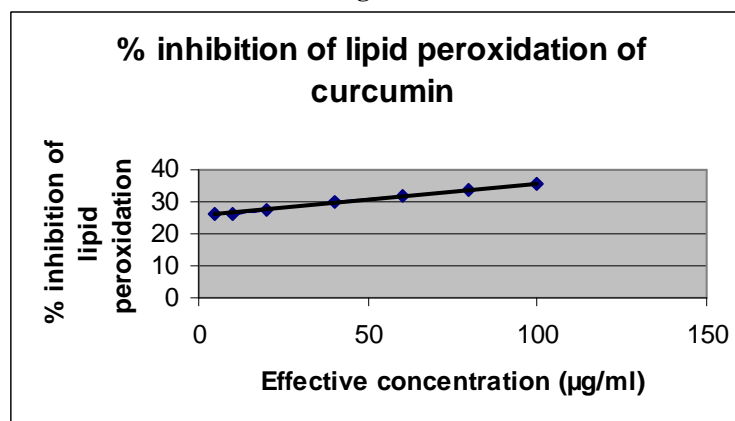
MECA seeds showed promising free radical scavenging effect of DPPH in concentration dependent manner upto 100 mg/ml. The IC_{50} values of standard drug and plant part were found to be 24.67mg/ml and 39.17mg/ml whereas R^2 was found to be 0.9955 and 0.9943. (Table no.5 and 6, Figure no.5 and 6).

Figure 3



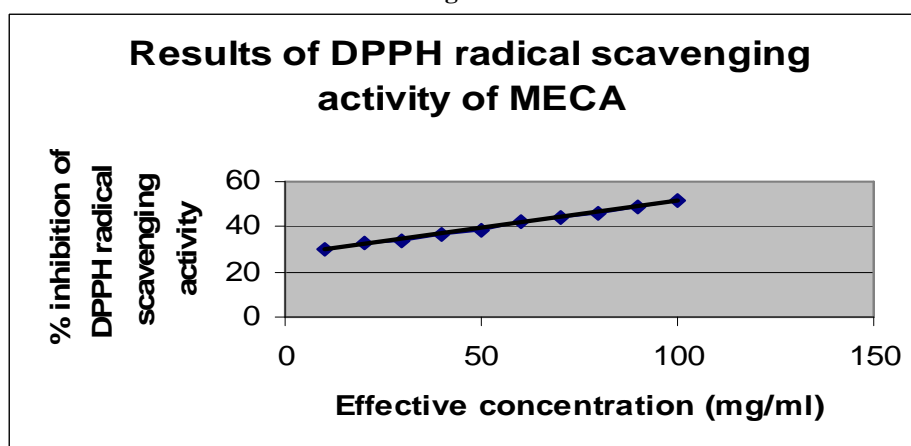
Equation for the line for the linear segment of the curve
 $Y=0.3954x+24.216$ and $R^2=0.9955$
 $IC_{50}=43.99\mu\text{g/ml}$

Figure 4



Equation for the line for the linear segment of the curve
 $Y=0.1033x+25.505$ and $R^2=0.9979$
 $IC_{50}=30.67\mu\text{g/ml}$

Figure 5



Equation for the line for the linear segment of the curve
 $Y=0.2403x+27.156$ and $R^2=0.9943$

Table 4: Results of Lipid Peroxidation Inhibitory Activity of Curcumin

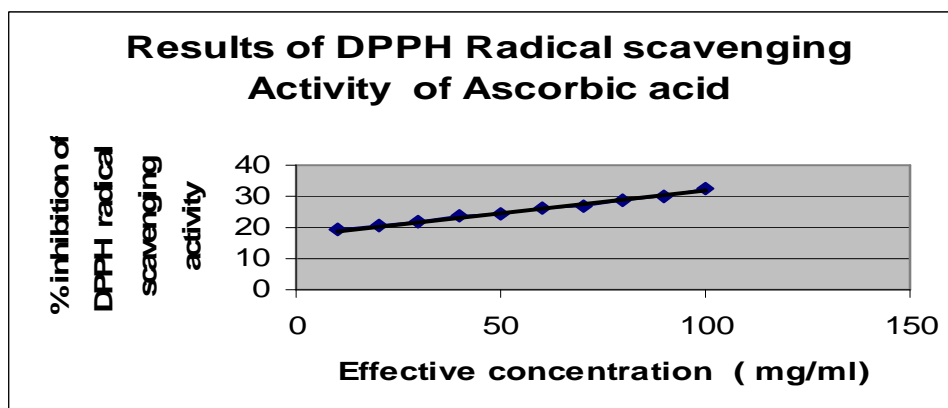
Effective concentration ($\mu\text{g/ml}$)	% Inhibition in TBARs formation	IC ₅₀ value
5	25.97	30.67 $\mu\text{g/ml}$
10	26.52	
20	27.37	
40	29.88	
60	31.90	
80	33.80	
100	35.65	

Table 5: DPPH Radical scavenging Activity of MECA

Effective concentration (mg/ml)	% inhibition of DPPH radical scavenging activity	IC ₅₀ value
10	30.17	39.17mg/ml
20	32.50	
30	34.00	
40	36.20	
50	38.00	
60	41.92	
70	44.12	
80	46.40	
90	48.90	
100	51.50	

Table 6: DPPH Radical scavenging Activity of Ascorbic acid

Effective concentration (mg/ml)	% inhibition of DPPH radical scavenging activity	IC ₅₀ value
10	19.07	24.67 mg/ml
20	20.40	
30	21.84	
40	23.50	
50	24.59	
60	25.95	
70	27.11	
80	28.75	
90	30.25	
100	32.46	

Figure 6

Equation for the line for the linear segment of the curve

$$Y = 0.1432x + 17.519 \text{ and } R^2 = 0.9955$$

$$\text{IC}_{50} = 24.67 \text{ mg/ml}$$

DISCUSSION AND CONCLUSION

Sodium nitroprusside serves as a chief source of free radicals. Scavengers of nitric oxide compete with oxygen leading to reduced formation of Nitric Oxide (NO). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethylene diamine is used as the marker for NO scavenging activity [9]. The chromophore formation was not complete in the presence of MECA, which scavenges the NO thus formed from the sodium nitroprusside and hence the absorbance decreases as the concentration of the MECA extract increases in a dose dependent manner [11].

Lipid peroxidation has been implicated in the pathogenesis of various diseases including arthritis. It is well established that bioenzymes are very much susceptible to LPO, which is considered to be the starting point of many toxic as well as degenerative processes. The MECA extract exhibited protection against lipid peroxidation induced by FeSO₄. Initiation of lipid peroxidation by ferrous sulphate takes place through Ferryl perferryl complex [10, 12]. The MECA inhibited the FeSO₄ induced lipid peroxidation in a dose dependant manner. The inhibition could be caused by the inhibition of formation of Ferryl perferryl complex. The presence of flavonoids in *Cassia auriculata* flowers may be responsible for antioxidant activities.

The DPPH system is a stable radical generating procedure [13]. It is well known that the DPPH has ability to capture free radicals is due to the delocalization of the unpaired electron all over the molecule. DPPH is a potent scavenger for many other radicals, due to the easiness in following the procedure – violet colour of DPPH faints into the yellow colour of its reduced congener (DPPH-H), with a high shift in the visible spectra (from 520 nm to 330nm) [14].

Cassia auriculata is widely used in number of pharmacological actions with high content of flavanoids and bioflavonoid seems to have a high potential for antioxidant activity. Thus plants each and every part has got its utility.

Acknowledgements

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