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In-vitro antioxidant potential of the flavonoid glycosides from *Cassia tora* linn. Leaves

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Abstract - The present study aimed to study the anti-oxidant potential of isolated flavonoids from the ethanol extract (70%v/v) of Cassia tora leaves. Three flavonoids were isolated viz Luteolin-7-O-β-glucopyranoside (I), quercetin-3-O-β-d-glucuronide (II) and Formononetin-7-O-β-D-Glucoside (III) from the ethanol extract (70%v/v) of Cassia tora leaves in the previous study. A comprehensive study on the phytochemical contents - total phenolic content and flavonoid content was accessed using Folin-Denis and $AlCl_3$ method respectively. The antioxidant potential of the samples was evaluated using inhibition of hydroxyl radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and nitric oxide scavenging methods. The total phenolic content and flavonoid content was found to be 18.60 % w/w and 9.5% w/w respectively. The IC_{50} values of ethanol extract against hydroxyl, DPPH and nitric oxide radical were found to be 270 µg/mL, 190 µg/mL and 130 µg/mL respectively. The radical scavenging activity of the isolated flavonoids decreased in the following order: quercetin (IC_{50} values 15, 14, 18 µg/mL) >formononetin (IC_{50} values 19, 21, 14 µg/mL) > luteolin (IC_{50} values 20, 23, 18 µg/mL) respectively.

Index terms - Cassia tora, flavonoids, HPTLC, antioxidant

I. INTRODUCTION

Free radicals, chemical reactions and several redox reactions of various compounds may cause protein oxidation, DNA damage and lipid peroxidation in living cells [1[. Therefore, oxidation have been claimed to play an important role in human health and causing several diseases, including cancer, hypertension, heart attack and diabetes [2[. However, living organisms have developed antioxidant systems to counteract reactive species and to reduce their damage. These complex antioxidant systems include enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) macromolecules and an array of small molecules, including ascorbic acid, α-tocopherol, carotenoids, polyphenols, uric acid and bilirubin. Oxidative damage occurs when this system is overwhelmed. Antioxidants could increase these complex antioxidant systems and protect the human body against free radicals that may cause pathological conditions, such as anaemia, arthritis, inflammation, neurodegeneration, ageing process and perhaps dementias [3]. Free radicals can be scavenged through utilizing natural antioxidant compounds present in medicinal plants. Some medicinal plants have been shown to have both chemopreventive and/or therapeutic effects on human diseases [4].

Many antioxidant compounds from plant sources have been identified as free radical or active oxygen scavengers. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in food or medicine to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity [5]. Natural antioxidants can protect the human body from free radicals and arrest the progress of many chronic diseases as well as retard lipid oxidative rancidity in food

Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers. Flavonoids are important for human health because of their high pharmacological activities as radical scavengers. Flavonoids possess various clinical properties such as antioxidant, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic activities; while some flavonoids exhibit potential for anti–human immunodeficiency virus functions [6]. This activity is attributed to their hydrogen donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available ‘‘H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure. Free radical

scavenging capacity is primarily attributed to high reactivities of hydroxyl substituents that participate in the reaction [7].

$$\mathbf{F\text{-}OH + R^{\cdot} \rightarrow F\text{-}O^{\cdot} + RH}$$

Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical, thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain [8].

Cassia tora Linn. (Family : Caesalpiniaceae) is an annual herb, 30-39 cm high growing in India as wasteland rainy season weed. The leaves and seeds of *Cassia tora* are found to be used in leprosy, psoriasis, ring worm, flatulence, colic, dyspepsia, constipation, cough, bronchitis and cardiac disorders in the Ayurvedic system of medicine. It is a rich source of anthraquinone glycosides and flavonoids [9,10]. Hence the present study was designed to evaluate antioxidant activity of flavonoids isolated from the ethanol extract of *Cassia tora* leaves.

II. METHODOLOGY

Three flavonoid glycosides were isolated viz Luteolin-7-O-β-glucopyranoside (**I**), quercetin-3-O-β-d-glucuronide **(II)** and Formononetin-7-O-β-D-Glucoside **(III**) from the ethanol extract (70%v/v) of *Cassia tora* leaves in the previous study [11].

2.1 Estimation of Phytoconstituents

The phytoconstituents present in dried coarsely powdered *Cassia tora* leaves was estimated using standard procedures.

2.1.1 Total Phenolic Content

The content of total phenolics in the powdered drug was determined by using Folin-Denis reagent. About 1 gram of the powder was extracted in an ultrasonic wave bath with 80 ml of aqueous ethanol solution (70% v/v) for 2 hr. After cooling, the volume of the solution was adjusted to 100 ml. The final solution was centrifuged prior to the colorimetric determination. Tannic acid standards (10 - 110 mg/ml) were dissolved in 100 ml of aqueous ethanol solution (70% v/v) respectively. About 10 ml of Folin-Denis reagent was added to 1 ml of the extract solution and 1 ml of standard solution. After reacting for 3 min, 10 ml of 35% sodium carbonate solution was added and the test solution was diluted to 100 ml with water and mixed. After 45 min, an aliquot was centrifuged for 5 min. The absorption coefficient for the supernatant was measured at 745 nm. The total phenolic content of the extract were calculated using the mean regression coefficient from the standards [12].

2.1.2 Determination of Total Flavonoids

Total flavonoid content in dried plant material was estimated by spectrometric method. (Perkin-Elmer UV-Vis spectrometer Lambda 16 (Germany)) [13]. Dried powdered plant material (10 gm) was extracted by continuous mixing in 100 ml of 70% ethanol, 24 hr at room temperature. After filtration, ethanol was evaporated untill only water remained. Water phase was subsequently extracted with ethyl acetate. The extract was dried over anhydrous sodium sulphate, filtered and concentrated under vacuum up to a concentration of 1 gm/ml of extract. They were further diluted with ethyl acetate to obtain 0.01 gm/ml solutions. About 10 ml of the solution was transferred into a 25 ml volumetric flask, 1 ml of 2% $AlCl_3$ was added and the solution was filled to volume with methanol-acetic acid and was kept aside for 30 min. The absorbance was measured at 390 nm against the same solution without $AlCl_3$ being blank. The total flavonoid content was determined using a standard curve with quercetin (100 - 1000 mg/L) as the standard. Total flavonoid *content is expressed as mg of quercetin equivalents (Q) /g of extract.*

2.2 HPTLC analysis with known marker

The ethanolic extract of *Cassia tora* leaves was further subjected to HPTLC for the conformation of the active constituents. HPTLC was performed on 10 cm × 10 cm aluminum backed plates coated with silica gel 60F254 (Merck, Mumbai, India). Standard solution of the markers Luteolin, Quercetin and Formononetin and sample solution were applied to the plates as bands 8.0 mm wide, 30.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by the use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-µL Hamilton (USA) syringe [14].

Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), with Toluene: Ethyl acetate: Formic acid (5 : 4: 1) (v/v/v), as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried with a hair dryer and then scanned at 254 nm with a Camag TLC Scanner with WINCAT software, using the deuterium lamp. A stock solution of standards Luteolin, Quercetin and Formononetin (100µg/mL) were prepared in methanol. Different volume of stock solution 1, 2, 3, 4 and 5µL, were spotted on to TLC plate to obtained concentration 100 ng, 200 ng, 300 ng, 400 ng and 500 ng/spot of Luteolin, Quercetin and Formononetin. Calibration curve range (100 - 500 ng/spot) for Luteolin, Quercetin and Formononetin was found to be linear.

2.3 In-vitro antioxidant

Several concentrations ranging from 50-400 µg/ml of the ethanolic extract and concentrations ranging from 5-40 µg/ml of flavonoids Luteolin-7-O-β-glucopyranoside (**I**), quercetin-3-O-β-d-glucuronide **(II)** and Formononetin-7-O-β-D-Glucoside **(III**) were tested for their antioxidant activity in different *in-vitro* models. It was observed that free radicals scavenging property of the test was found to be in a concentration dependent manner in all the models.

2.3.1 Nitric oxide radical scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction. Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025 M, pH: 7.4) was incubated with different concentrations of ethanolic extract (50-400 µg/ml) dissolved in phosphate buffer saline (0.025 M, pH: 7.4) and the tubes were incubated at 25^{o}C for 5 hr. Control experiments without the test compounds but equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% O-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The procedure was repeated with isolated flavonoids (5-40 µg/ml). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylene diamine was read at 546 nm [15]. All determinations were performed in 6 replicates. Percentage inhibition was calculated by using the formula,

$$\text{Percentage inhibition (\%)} = \frac{\text{Absorbance of control - Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.3.2 DPPH radical scavenging activity

The free radical scavenging activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH. About 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the different concentration of ethanolic extract (50-400 µg/ml), in different test tubes. The procedure was repeated with isolated flavonoids (5-40 µg/ml), and control (without the test compound, but with an equivalent amount of methanol). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer [16]. Decrease in absorbance of the reaction mixture indicates higher free radical scavenging activity.

2.3.3 Hydroxyl radical scavenging activity

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of ferric chloride, 0.1 ml of hydrogen peroxide, 0.36 ml of deoxyribose, 1 ml of ethanol extract (50-400 µg/ml), 0.33 ml of phosphate buffer (50 mM. pH 7.4), 0.1 ml of ascorbic acid in sequence and incubated at 37^{o}C for 1 hr. The procedure was repeated with isolated flavonoids (5-40 µg/ml), and control (without the test compound, but with an equivalent amount of methanol). A 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% tri chloro acetic acid and 1.0 ml of 0.5% thio barbituric acid to develop the pink chromogen, which was measured at 532 nm [17].

2.4 Statistical analysis

Level of significance of all the parameters was expressed as the arithmetic mean ± SEM and was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's "t" test. *P* value less than 0.05 ($P < 0.05$) was the critical criterion for statistical significance.

III. RESULTS AND DISCUSSION

The therapeutic potential of flavonoids and the necessity for scientific validation in popular medicine have prompted increased interest in the field..

3.1 Estimation of Phytoconstituents

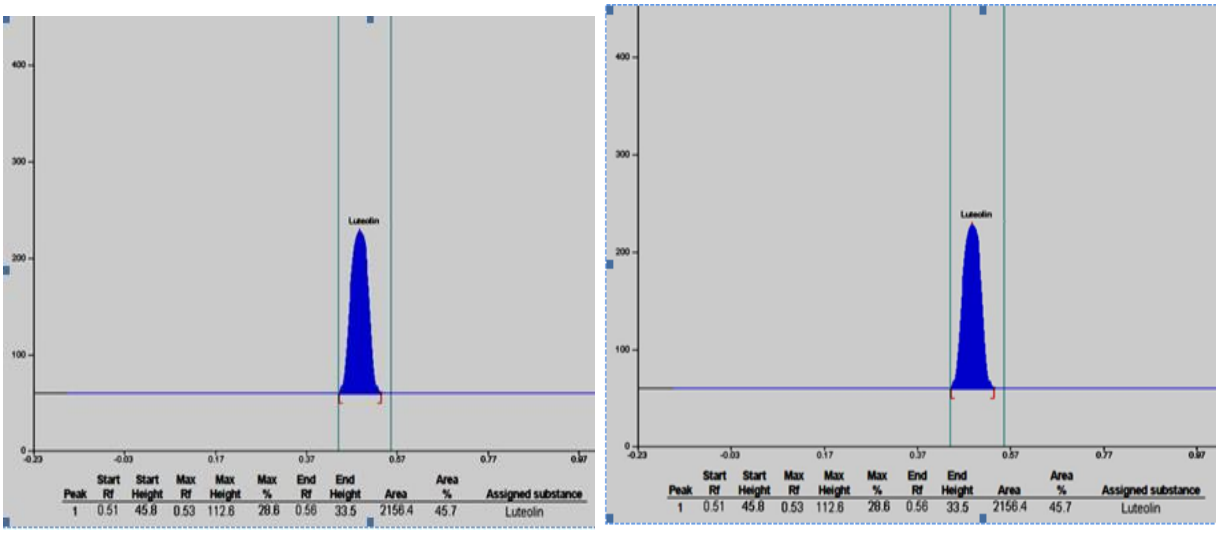
Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. The contents of total phenols and flavonoids were estimated by the standard curves and expressed as gallic acid equivalents for total phenols and quercetin equivalents for flavonoids. The amount of total phenolic content and total flavonoid content in the ethanol extract showed 18.60 mg/g dry weight, expressed as Gallic acid equivalents and 9.5% mg/g expressed as quercetin equivalents respectively.

3.2 HPTLC Fingerprint analysis with known marker

High performance thin layer chromatography (HPTLC) is an invaluable quality assessment tool for the evaluation and quantification of botanical materials. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of marker compound and mobile phase with very less time [18].

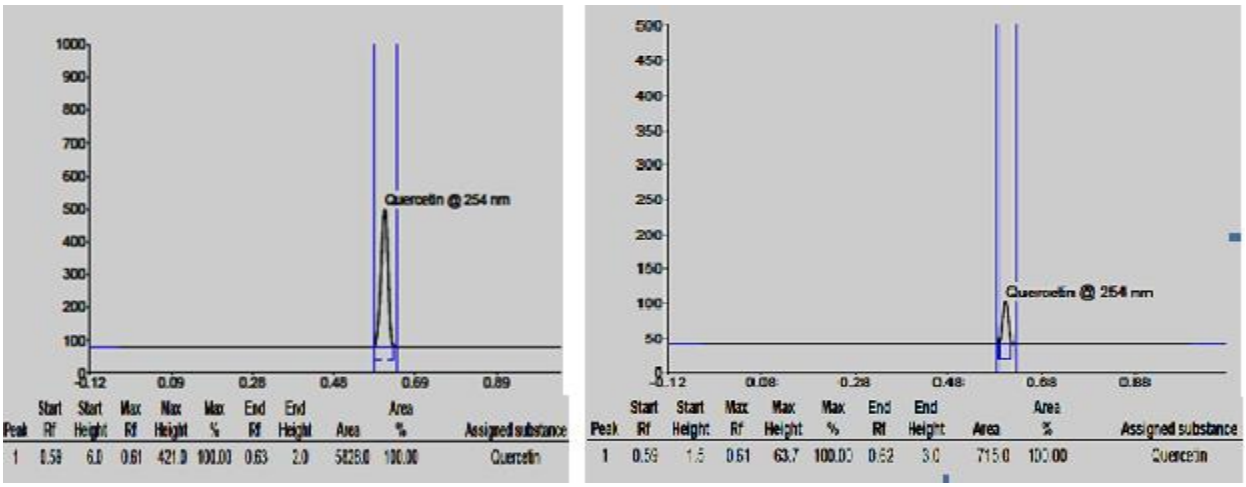
In the present study, the flavonoids Luteolin, Quercetin and Formononetin were detected and quantified using HPTLC silica gel 60 F254 pre-coated plates with the mobile phase made of Benzene: Methanol: Ammonia (90:10:1). Solvent systems were optimized to achieve best resolution of the marker compounds from the other compounds of the sample extracts. Of the various solvents tried Benzene: Methanol: Ammonia (90:10:1) gave best resolution for Luteolin (Rf 0.53), Quercetin (Rf 0.61) and Formononetin (Rf 0.76). The identity and purity of the bands in the sample extracts were confirmed by comparing the Rf values.

The identity of Luteolin, Quercetin and Formononetin in sample chromatograms was confirmed by comparison of the chromatogram obtained from the reference standard solution at various concentration 100-500 ng/spot at wave length 254 nm were shown in Figure 1-3. Amount of present in the ethanol extract was calculated from the calibration curve. The detection of Luteolin, Quercetin and Formononetin was observed to be linear over a concentration range of 100-500 ng/mL and the concentration of Luteolin, Quercetin and Formononetin was found to be Luteolin 220 ng/mg, Quercetin 160 ng/mg and Formononetin 210 ng/mg.



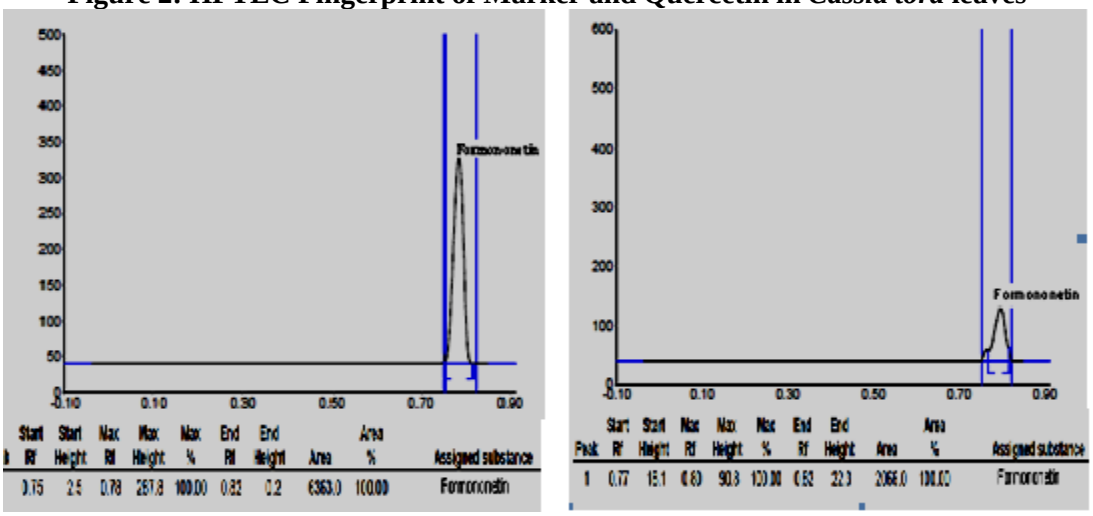
Marker **Ethanol extract**

Figure 1: HPTLC Fingerprint of Marker and Luteolin in *Cassia tora* leaves



Marker **Ethanol extract**

Figure 2: HPTLC Fingerprint of Marker and Quercetin in *Cassia tora* leaves



Marker **Ethanol extract**

Figure 3: HPTLC Fingerprint of Marker and Formononetin in *Cassia tora* leaves

3.3 Anti-oxidant activity

Antioxidants are significant in the prevention of human illness and may function as free radical scavengers, complexes of pro-oxidant metals, reducing agents and quencher of singlet oxygen formation [19]. Free radicals posses the ability to reduce the oxidative damage associated with many disease including neurodegenerative diseases, cancer, cardiovascular disease, cataracts and AIDS [20]. Antioxidants through their scavenging power are useful for the management of these diseases.

3.3.1 Nitric oxide scavenging activity

The scavenging of nitric oxide by ethanolic extract, and isolated flavonoids, were concentration dependent. The IC_{50} value of ethanolic extract, standard and isolated flavonoids **I, II, III** being of 130 µg/mL, 15 µg/mL, 18 µg/mL, 18 µg/mL and 14 µg/mL respectively. On a comparative basis, the flavonoid formononetin showed better activity in quenching nitric oxide with an IC_{50} value of 14µg/ml.

Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases. Nitric oxide is a very unstable species under aerobic conditions. It reacts with O^2 to produce stable product nitrate and nitrite through intermediates NO_2, N_2O_4 and N_3O_4. It is estimated by using Griess reagent and in presence of test compound which is a scavenger that the amount of nitrous acid will decrease [16]. In the present study the nitrite produced by the incubation of solutions of sodium nitro prusside in standard phosphate saline buffer at 25° C was reduced by the ethanol extract and isolated flavonoids. Significant

scavenging activity was observed for isolated flavonoids. This may be due to the antioxidant principle flavonoid, which competes with oxygen to react with nitric oxide, leading to reduced production of nitric oxide.

3.3.2 Inhibition of DPPH radical

The potential decrease in the concentration of DPPH radical due to the scavenging ability of ethanolic extract and isolated flavonoids **I, II, III** showed significant free radical scavenging activity of about 97%, 85%, 98%, and 90% respectively at higher doses with the IC_{50} value being 190 µg/ml, 23 µg/mL, 14 µg/mL and 21 µg/mL respectively.

DPPH assay is considered a valid and easy way to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to generate as in other radical assays. DPPH radicals react with suitable reducing agents and then electrons become paired off and the solution loses colour with the number of electrons taken up [16]. Such reactivity has been widely used to test the ability of compound and plant extract to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm. Thus an antioxidant candidate which proves promising in the DPPH antioxidant assay would provide an optimistic scaffold for prospective in vivo studies. Prasad et al [21]. reported that phenolics and flavonoids reduce the DPPH radical by their hydrogen donating ability.

3.3.3 Inhibition of Hydroxyl radical

The potentially reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The effect of ethanolic extract and isolated flavonoids on the inhibition of free radical-mediated deoxyribose damage was assessed by means of iron (II)-dependent DNA damage assay, which showed significant results. The IC_{50} value of ethanolic extract, standard and isolated flavonoids **I, II, III** being of 270 µg/mL, 18 µg/mL, 20 µg/mL, 15 µg/mL, and 19 µg/mL respectively. The results were tabulated in Table 1.

Table 1: Free radical scavenging activity of ethanol extract and flavonoids from *Cassia tora* leaves

Drugs	Concentration (µg/ml)	Hydroxyl radical inhibition (%)	DPPH radical inhibition (%)	Nitric oxide inhibition (%)
Ethanol extract	50	11.24±1.14	13.36±1.36	14.14±1.29
	100	18.45±1.72	28.26±2.14	30.36±1.40
	200	39.13±1.32	56.25±1.46**	61.02±1.72**
	300	54.24±0.66**	80.14±2.52**	82.31±1.79**
	400	83.24±1.36**	97.17±2.40**	94.15±1.50**
	IC_{50}	**270 µg/ml**	**190 µg/ml**	**130 µg/ml**
Compound **I**	5	12.68±1.20	11.97±1.67	12.20±1.58
	10	26.46±1.89	22.24±0.05	27.21±1.69
	20	50.90±2.11**	44.90±1.12	54.35±1.50**
	30	74.43±1.45**	68.15±0.93**	69.12±1.40**
	40	88.16±2.36**	85.36±3.17**	90.14±0.75**
	IC_{50}	**20 µg/ml**	**23 µg/ml**	**18 µg/ml**
Compound **II**	5	19.32±0.08	18.43±1.22	11.23±1.36
	10	33.48±1.64	34.21±1.09	28.32±2.20
	20	68.40±2.60**	69.68±0.83**	57.28±1.27**
	30	80.20±3.02**	85.35±1.60**	82.43±1.47**
	40	96.27±1.03**	98.08±0.94**	93.27±2.38**
	IC_{50}	**15 µg/ml**	**14 µg/ml**	**18 µg/ml**
Compound	5	13.06±1.46	10.90±1.48	
	10	24.41±2.10	21.69±2.13	19.24±1.37

III	20 30 40 **IC_{50}**	52.45±1.60** 79.08±2.20** 87.32±3.28** **19 µg/ml**	48.83±1.56** 72.56±3.22** 90.54±1.38** **21 µg/ml**	34.42±2.15 68.11±3.20** 80.25±0.36** 95.68±2.59** **14 µg/ml**
Ascorbic acid	5 10 20 30 40 **IC_{50}**	14.31±1.20 30.46±3.09 59.68±1.38** 88.25±1.49** 94.60±2.30** **18 µg/ml**	12.38±1.80 20.68±1.28 41.36±1.82 62.84±1.82** 80.82±2.18** **24 µg/ml**	10.81±1.72 18.68±0.62 28.26±2.31 44.36±1.28** 70.10±2.16** **35 µg/ml**

Values are mean ± SEM of 6 parallel measurements. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test (n=6). All the values are statistically significant at $**P< 0.01$.

IV. Conclusion

In present experiment, we conceded out a systematic record on the relative free radical scavenging activity in ethanolic extract of the leaves of *Cassia tora*. The result of the present study revealed that the bark the leaves of *Cassia tora*, which hold maximum amount of flavonoid and phenolic compounds, exhibited the effective antioxidant activity.

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