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EVALUATION OF *IN VITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF *CASSIA AURICULATA* LINN. EXTRACTS

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ABSTRACT: Objective: To evaluate *in vitro* antioxidant and anti-inflammatory activity of *Cassia auriculata* leaf extracts. **Methods:** Herbal extraction was done by Soxhlet extraction method with increasing polarity of Solvents viz., Chloroform, Ethanol, Methanol. Phytochemicals analysis was done using different biochemical tests. Antioxidant potential of plant extracts were analyzed by ferric ion reducing antioxidant power, phosphor-molybdenum and 2, 2-diphenyl-1-picrylhydrazyl, and anti-inflammatory activity by using protein denaturation *in vitro* bioassay. Total phenolic content of each extract was also determined to assess their corresponding effect on antioxidant capacity of plant. **Results:** Phytochemicals analysis showed that each solvent extract contained broad spectrum of secondary metabolites, phenolic compounds, flavonoids, tannins and glycosides, where as compared to other solvent extracts, chloroform extract showed little quantity of phenolic Compounds, methanol extract exhibited the highest phenolic content and the significant Antioxidant capacity based on the test performed. Out of all extracts, methanol extract showed high anti-inflammatory activity. **Conclusion:** The present study revealed that different solvent extracts of *Cassia auriculata* leaves contain broad spectrum of bioactive compounds. Results confirm that methanol extract exhibited high antioxidant activity and anti-inflammatory activity.

INTRODUCTION: Oxidative stress is an imbalance in the redox status of a cell and the excessive production of free radicals can lead to damages, mutations and ultimately the formation of cancer. Ionizing radiation exposure can impact human health in different ways and cause a broad spectrum of adverse effects including anti-proliferative, pro-inflammatory and other biological effects.

These effects are mainly due to oxidative stress induced by irradiation. Defense against oxidative stress is therefore very important in preventing the development of diseases.

Many of the functional Phytochemicals are ascribed to their biological active poly phenol components, such as anthocyanins, flavonoids and phenolic acids, which have been proved to possessing powerful antioxidant activities. They are usually associated with prominent biological properties and prevention damage of cellular molecules. Evaluation of the total antioxidant capacity of plant products cannot be performed accurately by any single method due to their complex nature¹.

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Generally, for the extraction of the bioactive compounds from plant materials, water and some organic solvents such as ethanol, methanol, acetone and diethyl ether are usually used. Additionally, during the extraction process, the extract ability depends mainly on the types of solvent and the extraction methods². Therefore, the objective of the study was to investigate the soluble Phytochemicals components and *in vitro* antioxidant activities, including FRAP, DPPH and PM scavenging activities of *Cassia auriculata* extracts using various solvents (Methanol, Ethanol and Chloroform extract). Also Inflammation is a very common symptom of many chronic diseases and it is normal protective response to tissue injury caused by chemical or microbial agents³. It is well known fact that denaturation of tissue protein lead to inflammatory and arthritic diseases⁴.

Inflammation plays an important role in various diseases, such as rheumatoid arthritis and asthma. During an inflammatory response, mediator such as cytokines, interleukin-1, tumor necrosis factor and interferon's are released^{5,6}. Since ancient times, in various cultures worldwide, inflammatory disorders and related diseases have been treated with plants or plant derived formulations. An anti-inflammatory activity of several plant extracts and isolated compounds has already been scientifically demonstrated^{7,8}. On revising the reviews and the studies of the herb *Cassia auriculata* till the date, *in vitro* antioxidant activities in different antioxidants parameters have not been studied. Thus in the present study; an effort has been made to overview the anti oxidant and anti-inflammatory activity of *Cassia auriculata*.

MATERIALS AND METHODS:

Plant Collection: *C. auriculata* leaves were collected from the campus of Basaveshwar Science College, Bagalkot India in the month of June 2014. The leaves were identified and authenticated by Dr. S. A. Kappali, Department of Botany, Basaveshwar Science College, Bagalkot, Karnataka. A voucher specimen was deposited in the Department of Botany, Basaveshwar Science College, Bagalkot, Karnataka. Fresh plant material was washed under running tap water, air dried and then homogenized to fine powder. The powder was stored in airtight containers at - 20 °C for further use.

Crude Extraction: About 100g dried leaves were coarsely powdered and subjected to successive extraction by Soxhlet extractor. The extraction was done with different solvents in their increasing order of polarity such as Chloroform, Methanol and Ethanol. Each time the plant material was dried and later extracted with other solvents. All the extracts were concentrated by rotary vacuum evaporator and evaporated to dryness.

Phytochemicals Analysis: The crude powder of *C. auriculata* was qualitatively tested for different Phytochemicals constituents namely alkaloids, flavonoids, glycosides, phenols, lignins, saponins, sterols, tannins, anthraquinone and reducing sugar by following the standard procedure of Deepti *et al.*,⁹.

Estimation of Total Phenolic Content: The total phenolic content of the *C. auriculata* leaf extract was estimated by using Folin Ciocalteu method of Singleton *et al.*, with slight modification¹⁰. Gallic acid was used as the reference standard. A volume of 0.5mL of plant extract was mixed with 2mL of the Folin-Ciocalteu reagent (10 fold) and was neutralized with 4ml of sodium carbonate solution (8% w/v). The reaction mixture was incubated at room temperature for 30 min for color development. The absorbance of the resulting color was measured at 765nm using UV-vis spectrophotometer. The total phenolic contents were estimated from the linear equation of standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g Gallic acid equivalent (GAE).

Determination of Antioxidant Activity by using *in vitro* Methods:

Ferric Ion Reducing Antioxidant Power (FRAP) Assay: Ferric ions reducing power was measured according to the method of Oyaizu with a slightest modification¹¹. Methanol, Ethanol and Chloroform extract of *C. auriculata* in different concentrations ranging from 100µL to 500µL were mixed with 2.5mL of 20m mol/L phosphate buffer and 2.5mL (1% w/v) potassium ferricyanide, and then the mixture was incubated at 50 °C for 30 min. Afterwards, 2.5mL of (10% w/v) trichloroacetic acid and 0.5mL of (0.1% w/v) ferric chloride were added to the mixture, which was kept aside for 10 min. Finally, the absorbance was measured at

700nm. Ascorbic acid was used as positive reference standard.

Phosphomolybdenum (PM) Assay: Total antioxidant activity was estimated by PM assay using standard procedure of Prieto *et al.*,¹². Methanol, Ethanol and Chloroform extract of *C. auriculata* in different concentration ranging from 100µL to 500µL were added to each test tube individually containing 3mL of distilled water and 1mL of molybdate reagent solution. These tubes were kept incubated at 95 °C for 90 min. After incubation, these tubes were normalized to room temperature for 20 - 30 min and the absorbance of the reaction mixture was measured at 695nm. Ascorbic acid was used as reference standard.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Ability Assay: The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of hydrogen donating antioxidant due to the formation of the non radical form DPPH-H¹³. The free radical scavenging activity of all the extract was evaluated by 2, 2- diphenyl- 1- picryl-hydrazyl (DPPH) according to the previously reported method (Blois MS. Antioxidant determination by the use of stable free radical. Nat1958; 181: 1199-2000). Briefly, an 0.1mm solution of this solution was added to 3ml of the solution of all extract in methanol at different concentration (62.5, 125, 250 and 500µg/ml).

The mixture were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance were measured at 517nm using a UV-vis spectrophotometer. Ascorbic acid was used as reference. Lower values of the reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

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

Where, A₀ is the absorbance of the control reaction, and A₁ is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Evaluation of *in vitro* Anti-inflammatory Activity: Anti-inflammatory activity of methanol, ethanol and chloroform extract of *C. auriculata* was evaluated by protein denaturation method as described by Padmanabhan *et al.*, with slight modifications¹⁴. Diclofenac sodium, a powerful non steroidal anti-inflammatory drug was used as a standard drug. The reaction mixture consisting of 2mL of different concentrations of *C. auriculata* methanol, chloroform and ethanol extract (100 - 500µg/mL) or standard Diclofenac sodium (100 and 200µg/mL) and 2.8mL of phosphate buffered saline (pH 6.4) was mixed with 2mL of egg albumin (from fresh hen's egg) and incubated at (27 ± 1) °C for 15 min. Denaturation was induced by keeping the reaction mixture at 70 °C in a water bath for 10 min. After cooling, the absorbance was measured at 660nm by using double distilled water as blank. Each experiment was done in triplicate and the average was taken. The percentage inhibition of protein denaturation was calculated by using the following formula;

$$\text{Inhibition (\%)} = \frac{A_t - A_c}{A_c} \times 100$$

Where, A_t is absorbance of test sample, and A_c is absorbance of control.

RESULTS:

Phytochemicals Analysis: Preliminary Phytochemicals analysis of the extract showed the presence of major classes of Phytochemicals such as Tannins, alkaloids, flavonoids, cardiac glycosides *etc.*, (**Table 1**). Proteins and amino acids were not detected in these extract.

TABLE 1: PHYTOCHEMICALS CONSTITUENTS OF *C. AURICULATA* LEAF EXTRACT

Phytochemicals	Test	Ethanol Extract	Methanol Extract	Chloroform Extract
Alkaloid	Iodine, Wagnore's, Dragandroff's	+, +, -	+, +, -	+, +, -
Flavonoids	Shinoda Test, NaOH Test	+, ++	++, ++	+, ++
Glycosides	K, K test, Conc. H ₂ SO ₄ Test, Molisch Test	++, ++, ++	++, ++, ++	++, ++, ++
Phenols	Phenol Test	++	++	++
Lignins	Lignin Test	-	-	-

Saponins	Foam Test, Hemolysis Test	+, +	+, +	+, +
Sterols	Salkowski Test	+	+	+
Tannins	Gelatin Test, Lead acetate Test	++, ++	++, ++	+, +
Antraquinone	Bomtrager's test	-	-	-
Reducing sugar	Reducing Sugar test	-	-	-

Total Phenol Content: In the present study, total phenolic content of different extracts of leaves of *C. auriculata* determined by the Folin-Ciocalteu reagent¹⁵ and expressed as GAE per gram of plant extract. Ethanol extract exhibited the highest amount of phenolic content among the extracts, i.e. (68.25 ± 0.14) mg/g GAE followed by (67.16 ± 0.07) mg/g GAE in methanol extract (60.91 ± 0.14) mg/gGAE in chloroform extract. Variation was observed in total phenolic content.

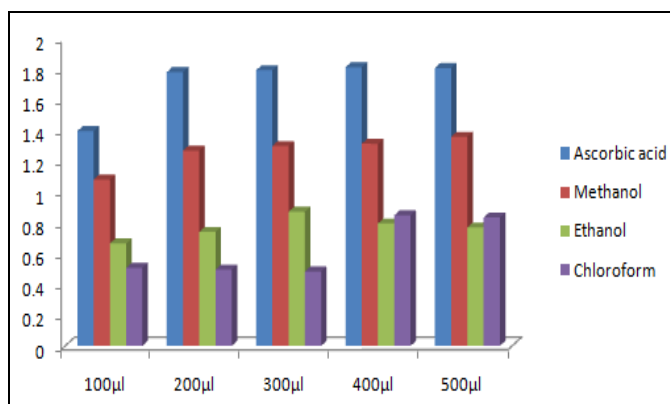


FIG. 1: FRAP ASSAY OF CASSIA AURICULATA L. EXTRACTS

FRAP Assay: In the present study, Methanol, Ethanol and Chloroform extracts were subjected to FRAP assay along with standard Gallic acid. In the results obtained, Methanol extract showed higher activity than the Ethanol and Chloroform extract

(**Fig. 1**) which was comparable to standard ascorbic acid.

PM Assay: In the present study, Methanol, Ethanol and Chloroform extracts were subjected to PM assay along with standard ascorbic acid. Ethanol extract showed the highest activity among methanol extract and standard ascorbic acid (**Fig. 2**).

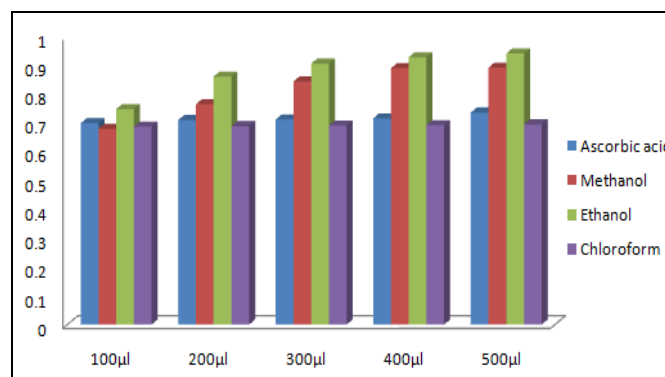


FIG. 2: PM ASSAY OF CASSIA AURICULATA L. EXTRACTS

DPPH Assay: In the present study, the different concentrations of Ethanol chloroform and methanol extract of leaves of *C. auriculata* were subjected to DPPH free radical scavenging assay. The antioxidant capacity of the extract was compared with ascorbic acid as standard as antioxidant. Ethanol extract exhibited higher antioxidant activity than the methanol extract (**Table 2**).

TABLE 2: PERCENTAGE INHIBITION OF DPPH FREE RADICAL OF C. AURICULATA EXTRACTS % CONCENTRATION INHIBITION

Concentration	Inhibition (in %)			
	Methanol Extract	Ethanol Extract	Chloroform Extract	Standard
62.5µg/ml	67.97±1.21	39.87±1.01	49.34±2.01	95.9±1.31
125µg/ml	80.83±0.98	56.02±1.35	59.58±1.45	96.85±1.12
250µg/ml	86.61±0.85	70.06±0.85	73.75±0.68	97.11±0.24
500µg/ml	90.02±1.21	77.26±1.24	74.85±0.58	97.63±0.35
1000µg/ml	91.07±0.96	82.23±1.58	81.36±0.96	98.95±0.86

In vitro Anti-inflammatory Assay: In the present study, known concentrations of Ethanol, chloroform and methanol extract of *C. auriculata* were subjected for anti inflammatory activity on protein denaturation. The *in vitro* anti inflammatory activity of the extract was comparable to the Diclofenac sodium, a reference drug¹⁴. A

significant difference in the inhibition of thermally induced protein denaturation was observed in case of Ethanol, Chloroform and Methanol extract when compared with standard drug at concentration of 100µg/mL. Ethanol and Methanol extract shows higher inhibition activity whereas Chloroform extract showed less inhibitory activity (**Table 3**).

TABLE 3: IN VITRO ANTI-INFLAMMATORY EFFECT OF *C. AURICULATA* LEAF EXTRACTS %

Treatment	Concentration	Inhibition
Methanol Extract	100 µg/mL	78.15±0.98
Ethanol Extract	100 µg/mL	74.69±1.12
Chloroform Extract	100 µg/mL	73.26±1.02
Diclofenac sodium	100 µg/mL	76.73±1.36

DISCUSSION: Medicinal plants are since ancient times lauded for their diverse pharmacological actions which could be attributed to the presence of secondary metabolites such as alkaloids, flavonoids, glycosides, tannins, steroids *etc.* Some of these plants are important sources of natural antioxidant that have been shown to reduce the risk and progression of certain acute and chronic diseases such as cancer, heart diseases and stroke by scavenging free radicals which are impetrated in the pathogens us of many diseases^{16,17}.

The results of preliminary Phytochemicals screening confirmed the presence of various classes of Secondary metabolites in the *Cassia auriculata* leaf extract including polyphenols (Tannins and Flavonoids). Plant polyphenols produced either from phenyl alanine or its precursor shikmic acid, are important dietary antioxidants because they possess ideal structural chemistry for free radical scavenging activity. Numerous *in vitro* studies have shown antioxidant potential in protecting against many diseases¹⁸. The present study indicates that leaves of *Cassia auriculata* are rich in phenolic compound (68.25mg/g) with ethanolic extract.

The Antioxidant activity of the *C. auriculata* was assessed using FRAP and reducing power scavenging assays. The FRAP assay evaluates the ability of a substance to reduce Fe^{3+} to Fe^{2+} , which is measured by the formation of a coloured complex with TPTZ that can be read spectrophotometrically at 593nm. Since the antioxidant activity of a substance is usually correlated to its reducing capacity, this assay provides a reliable method to evaluate the antioxidant activity¹⁹.

The methanol extract of the herb was found to be having significant anti-oxidant activity. These results may also be helpful to describe the various pharmacological activities like anti-infective, protective activities.

The DPPH test provides information on the reactivity of the test compounds with stable free

radicals. The DPPH assay is often used to evaluate the ability of antioxidants to scavenge the free radical from test samples. Whereby, the free radicals cause biological damage through oxidative stress and such process lead to many disorders like nerodegenerative diseases, cancer and AIDS²⁰. Therefore, DPPH assay is an effective method to measure their scavenging power. The principle of the DPPH assay is based on the colour changes from purple (DPPH solution) to yellow²¹. The colour changes can be measured quantitatively by spectrophotometer absorbance at 517nm.

In the present study, antioxidant activity of *C. auriculata* was evaluated using Chloroform, Ethanol and Methanol extract of plant and was compared with standard ascorbic acid. Results obtained showed that standard antioxidant had higher scavenging activity at all tested concentrations than the extracts. Among the extracts, Methanol extract exhibited higher activity than the Ethanol and chloroform extract (**Table 2**). Significant difference was observed among antioxidant activities of evaluated extracts and methanol extract came out as a superior total antioxidant capacity, with significant higher results in all performed assays.

Inflammation is a symptom of many chronic diseases. It is a normal protective response to tissue injury caused by physical taurama noxious chemical or by microbial agents. Inflammation is a protective attempt by the body to remove injurious stimuli as well as initiate the healing process of the tissue²². It is non steroidal anti-inflammatory drugs are commonly used for the management of inflammatory conditions, but these are associated with many unwanted side effects such as gastric irritation, ulcer *etc.*,²³. Medicinal plants used in traditional medicine to treat anti-inflammatory conditions seem viable and logical alternative in search of safe and effective anti-inflammatory agent. Hence in this study plant extract subjected to simple and viable protein denaturation bioassay method to evaluate its potential as anti-inflammatory drug. It is well known fact that denaturation of tissue protein leads to inflammatory and arthritic diseases²⁴.

C. auriculata leaf extract and reference drug Diclofenac sodium exhibited dose dependent

percentage of inhibition eat induced protein denaturation in fresh egg albumin percent inhibition of protein denaturation with respect to control is a measure of protein stabilization²⁵. *C. auriculata* leaf extract effect on inhibition of protein denaturation was found to be comparable with the standard drug Diclofenac sodium it shows Satisfactory anti inflammatory activity. The results of our study suggest that *C. auriculata* leaf extract rich in phenolic compound and have a good anti oxidant activity.

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CONFLICT OF INTEREST: We declare that we have no conflict of interest.

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