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In vitro Free Radical Scavenging and Antioxidant Activity of *Cicer arietinum* L. (Fabaceae)

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Abstract: Extract and its different fractions of mature pod wall of *Cicer arietinum* Linn. (Family: Fabaceae) was assessed for its antioxidant activity by *in vitro* methods. Antioxidant activity was studied using 1, 1- Diphenyl-2-Picrylhydrazyl (DPPH), nitric oxide scavenging activity, hydrogen peroxide scavenging activity, reducing power assay. Antioxidant activities compared with ascorbic acid as standard antioxidant. Quantitative analysis of antioxidative components like total phenolic content, total antioxidant capacity were estimated using spectrophotometric methods. Results showed that extracts and fractions exhibited significant DPPH, nitric oxide and hydrogen peroxide activity. Total phenolic content and total antioxidant capacity of ethanol extract and ethyl acetate fraction was estimated. From results, it is concluded that flavonoids and related polyphenols present in *Cicer arietinum* extract may be responsible for the activity.

Key words: *Cicer arietinum,* antioxidant, DPPH, total phenolic content, total antioxidant capacity.

INTRODUCTION AND EXPERIMENTAL

INTRODUCTION

An antioxidant is a molecule capable of inhibiting or preventing the oxidation of other molecules. Antioxidants are substances that may protect cells from the damaging effects of oxygen radicals, highly reactive chemicals that play a part in atherosclerosis, some forms of cancer and reperfusion injuries. Medicinal plants are in important source of antioxidants. The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark[1].

The plant *Cicer arietinum* Linn. belonging to family Fabaceae, largely cultivated in most parts of India. Seed is aphrodisiac, anthelmintic, tonic, enriches the blood, cures skin diseases, inflammation; more especially of ear, diuretic[2], halitosis, hepatosis, otitis, pharyngosis, pulmonosis and splenosis[3], Ingredient of a Unani anti-hypertensive drug *Ajmaloon*[4]. Ingredient for preparation of *Nakhud*[5]. Acid exudation is astringent and useful in dyspepsia and constipation. Leaves are sour, astringent, improves taste and appetite, cures bronchitis, causes flatulence. Tart leaves are orexigenic, enterosis[2].

Isoflavonoids isolated from *Cicer arietinum* shows antifeedant activity[6]. Biochanin-A and formononetin isolated from *Cicer arietinum* were evaluated for management of diabetes mellitus[7]. Pangamic acid isolated from aqueous extract of *Cicer arietinum* has been evaluated for stamina building,

antistress, antihyperlipidimic activity[8]. The aqueous seed coat extract exhibited diuretic activity [9].

MATERIALS AND METHOD

Plant material

Mature pod walls of *Cicer arietinum* were collected from Chopda, Jalgaon, Maharashtra (India). During the month of April 2010. The plant was authenticated by Botanical survey of India, Pune, Maharashtra (India). A voucher specimen (No.CICAS3) has been kept in laboratory for future reference.

Preparation of plant extract

About 700 g of shade dried powder of pod wall of *Cicer arietinum* was successively extracted with petroleum ether and ethanol to obtain petroleum ether (PE) and ethanol extract (EE). The crude ethanol extract, after removal of solvent was partitioned with benzene, ethyl acetate and methanol successively to give benzene (BF), ethyl acetate (EAF) and methanol (MF) fractions respectively. Aqueous extract (AE) was prepared by making decoction of dried pod wall (500g) of *Cicer arietinum.*

Chemicals and reagent

DPPH obtained from Sigma Aldrich Ltd. Mumbai, ascorbic acid, sodium nitroprusside, sulphanilamide, phosphoric acid, α - naphthyl ethylene dihydrochloride, potassium dihydrogen phosphate, potassium ferricyanide, ferric chloride, tricholoroacetic acid, folin-Ciocateu's phenol reagent, ammonium molybdate were obtained from Loba Cheime Ltd. All other chemicals used were analytical grade.

Phytochemical evaluation

Extracts and fractions of *Cicer arietinum* were studied for its phytoconstituents such as carbohydrates, proteins, steroids, glycosides, saponin alkaloids, flavonoids, tannins and phenolic compounds using different phytochemical tests[10].

Antioxidant activity

Rapid screening of antioxidant compounds using TLC [11]

To make a semi-quantitative visualization possible, extracts and fractions of *Cicer arietinum* pod wall were applied on a TLC plate and developed in solvent system consisting of petroleum ether: ethyl acetate: methanol: water (3:7:3:1). After drying, the plate was sprayed with a 0.2% solution of DPPH in methanol and heated at 100^0 C for 5 minutes. Active compounds appeared as yellow spots against purple background, which is indirect measure of antioxidant activity.

DPPH radical scavenging assay[12, 13]

DPPH in methanol (0.004 %) was prepared and 1 ml of this solution was added to 1 ml of extract solution at different concentration (20-100 µg/ml). 30 minutes later, the absorbance was measured at 517 nm. A blank was prepared without adding extract. Ascorbic acid was used as standard. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation.

Percentage inhibition = $[A_0 – A_1 / A_0] X 100$

Where, A_0 absorbance of blank, A_1 absorbance of standard, extract or fraction

Nitric oxide scavenging assay[14]

The reaction mixture (3 ml) containing 10 mM sodium nitroprusside in phosphate buffer solution and the extract or reference compound (ascorbic acid) at different concentrations (20-100 µg/ml) were incubated at 25^0 C for 150 min. About 0.5 ml aliquot of the incubated sample was removed at 30 min. interval and 0.5 ml Griess reagent was added. The absorbance of the chromophore formed was measured at 546 nm. Inhibition of the nitric oxide generated was measured by comparing the absorbance values of control, extracts, fractions and ascorbic acid.

Percentage inhibition = $[A_0 – A_1 / A_0] X 100$

Where, A_0 absorbance of blank, A_1 absorbance of standard, extract or fraction.

Hydrogen peroxide scavenging assay[1]

A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM, pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using spectrophotometer. Extract (20-100 µg/ml) at different concentration was added to hydrogen peroxide and absorbance is determined after 10 min. against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenged is calculated using following formula.

Percentage H_2O_2 scavenged = $[A_0 – A_1 / A_0] X 100$

Where, A_0 absorbance of blank,
A_1 absorbance standard, extract or fraction

Reducing power assay[15]

1 ml of extract (20-100 µg/ml) prepared in water was mixed with (2.5 ml) phosphate buffer and (2.5 ml) potassium ferricyanide and incubated at 50^0 C for 20 min. thereafter (2.5 ml) trichloroacetic acid was added to the reaction mixture, which was then centrifuged at

3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with (2.5 ml) distilled water and (0.5 ml) freshly prepared ferric chloride. The absorbance was measured at 700 nm. A blank was prepared without adding extract. Ascorbic acid was used as standard. Increased concentration of reaction mixture indicates increase in reducing power.

Total phenolic content[16]

The Folin –Ciocalteu reagent assay was used to determine the total phenolics content. The sample (0.2 ml) of different concentration (100-500 µg/ml) was mixed with 2.5 ml of Folin- Ciocateu's phenol reagent and allowed to react for 5 min. Then 2 ml of sodium carbonate (7.5 %) solution was added and the final volume was made up to 10 ml with water. After 1 hr. of reaction at room temperature, the absorbance at 760 nm was determined. Tannic acid was used as standard for the calibration curve.

Total antioxidant capacity[17]

0.3 ml extract of different concentration (2-10 mg) was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95^0 C for 90 min. Then the absorbance of the solution was measured at 695 nm using spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract was used as blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Statistical analysis

Experimental results were mean ± SEM of three measurements. Analysis of variance was performed by ANOVA

RESULTS

Phytochemical evaluation

Phytochemical evaluation of EE and EAF revealed presence of carbohydrates, proteins, steroids, glycosides, flavonoids, tannins and phenolic compounds.

Rapid screening of antioxidant compounds using TLC

The intensity of the spots and reaction time of the DPPH radical scavenging capacities of the different extract and fraction of *Cicer arietinum* pod wall using the TLC method showed that EE and EAF gave highest antioxidant activities.

DPPH radical scavenging assay

Decrease in the concentration of DPPH radical was observed due to the scavenging ability of the extracts and fractions of *Cicer arietinum* pod wall. Scavenging ability decreased in the order of AA> EAF > MF> EE> BF> AE> PE which were statistical significant ($p<0.001$) compared to control (Figure 1). EE, EAF, BF showed maximum DPPH radical scavenging activity at concentration 80 µg/ml, while AE, MF and PE showed at 100 µg/ml

Nitric oxide scavenging assay

The extracts and fractions demonstrated ability to scavenge NO and inhibit nitrite formation generated *in-vitro* by sodium nitroprusside. The nitric oxide scavenging activity of extracts and fractions were in following order: AA> EAF > MF> BF> EE> AE> PE, significant with standard ($p<0.001$) (Figure 2). All extracts and fractions showed maximum nitric oxide scavenging activity at concentration 100 µg/ml.

Hydrogen peroxide scavenging assay

The H_2O_2 scavenging activity of extracts and fractions were in following order: AA> EAF > EE> BF> AE> MF> PE, significant with standard ($p<0.001$) (Figure 3). All extracts and fractions showed maximum nitric oxide scavenging activity at concentration 100 µg/ml.

Reducing power assay

The reducing power of bioactive extract and bioactive fraction was determined by potassium ferricynide reduction method. Reducing power of EE and EAF increased with increasing concentration (Figure 4). Reducing power of EE, EAF and ascorbic acid followed the order: AA> EAF> EE.

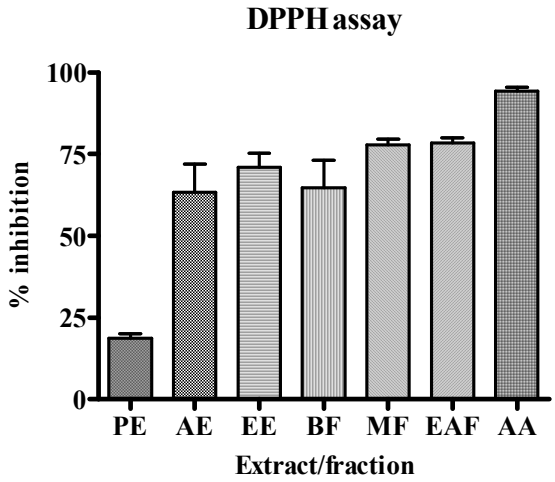
Total phenolic content

Phenolic content of bioactive fraction and bioactive extract was determined as tannic acid equivalent (Figure 5). Phenolic content of EE and EAF increased with concentration dependent manner. EE and EAF showed maximum phenolic content at 500 µg/ml concentration.

Total antioxidant capacity

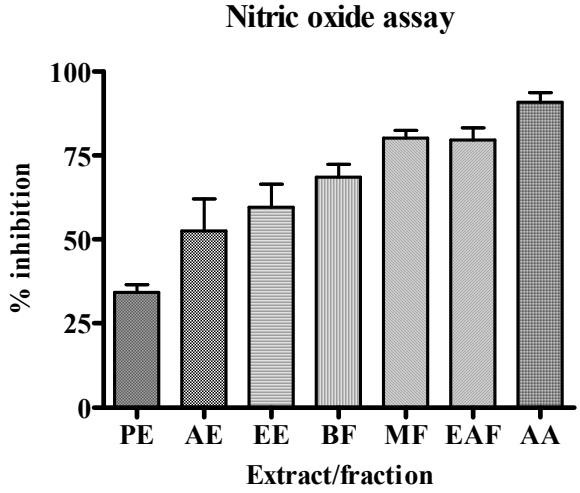
Total antioxidant capacity of bioactive fraction and bioactive extract was determined as ascorbic acid equivalent (Figure 6). Total antioxidant capacity of EE and EAF increased with concentration dependent manner. EE and EAF showed maximum total antioxidant capacity at 10 mg/ml concentration.

Figure 1: Free radical scavenging activity of extracts and fractions of pod wall of *Cicer arietinum* Linn. on DPPH



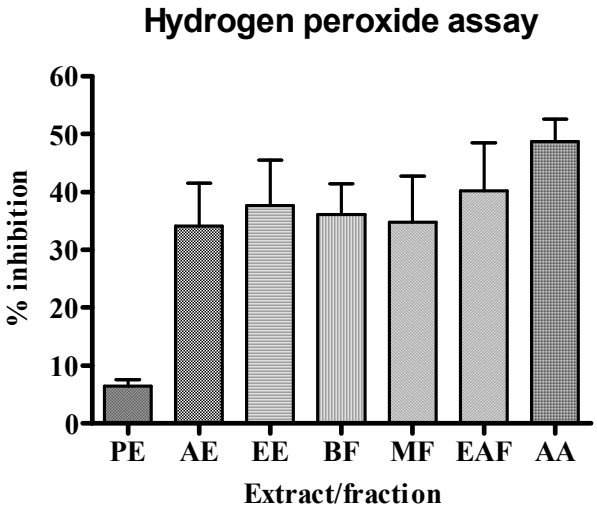
Values are expressed as mean± SEM, n=3
PE- Petroleum ether extract, AE- Aqueous extract, EE- Ethanol extract,
BF- Benzene fraction, MF- Methanol fraction, EAF- Ethyl acetate fraction,
AA- Ascorbic acid

Figure 2: Nitric oxide scavenging activity of extracts and fractions of pod wall of *Cicer arietinum* Linn.



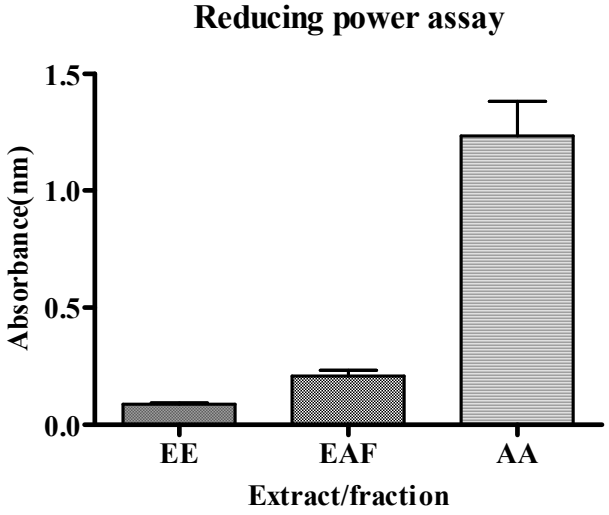
Values are expressed as mean± SEM, n=3
PE- Petroleum ether extract, AE- Aqueous extract, EE- Ethanol extract,
BF- Benzene fraction, MF- Methanol fraction, EAF- Ethyl acetate fraction,
AA- Ascorbic acid

Figure 3: Hydrogen peroxide scavenging activity of extracts and fractions of pod wall of *Cicer arietinum* Linn.



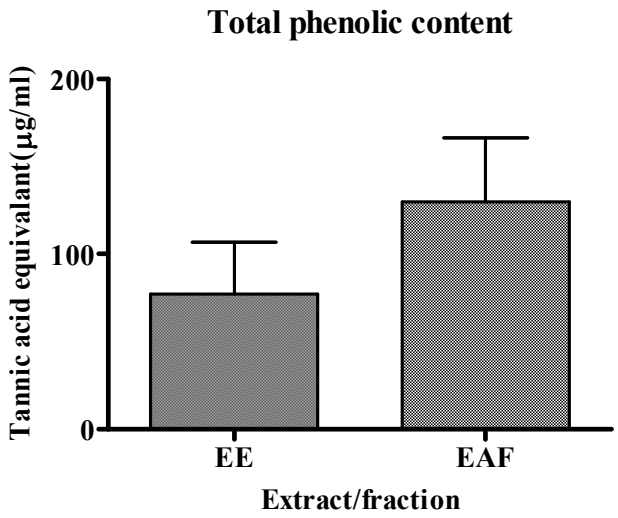
Values are expressed as mean± SEM, n=3
PE- Petroleum ether extract, AE- Aqueous extract, EE- Ethanol extract,
BF- Benzene fraction, MF- Methanol fraction, EAF- Ethyl acetate fraction,
AA- Ascorbic acid

Figure 4: Reducing power of extracts and fractions of pod wall of *Cicer arietinum* Linn.



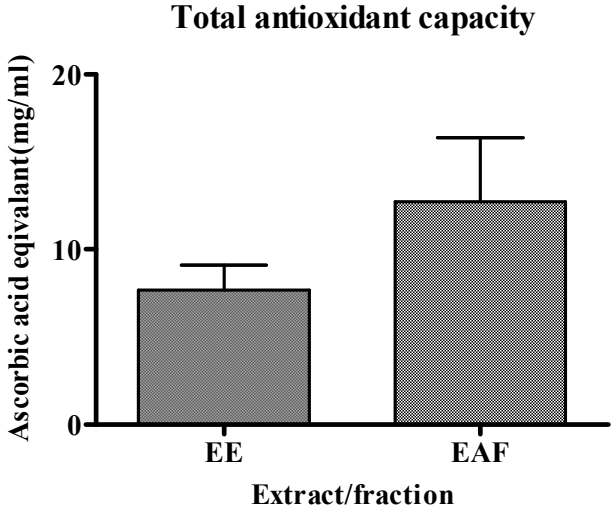
Values are expressed as mean± SEM, n=3
PE- Petroleum ether extract, AE- Aqueous extract, EE- Ethanol extract,
BF- Benzene fraction, MF- Methanol fraction, EAF- Ethyl acetate fraction,
AA- Ascorbic acid

Figure 5: Total phenolic content of extracts and fractions of pod wall of *Cicer arietinum* Linn.



Values are expressed as mean± SEM, n=3
PE- Petroleum ether extract, AE- Aqueous extract, EE- Ethanol extract,
BF- Benzene fraction, MF- Methanol fraction, EAF- Ethyl acetate fraction,
AA- Ascorbic acid

Figure 6: Antioxidant capacity of extracts and fractions of pod wall of *Cicer arietinum* Linn.



Values are expressed as mean± SEM, n=3
PE- Petroleum ether extract, AE- Aqueous extract, EE- Ethanol extract,
BF- Benzene fraction, MF- Methanol fraction, EAF- Ethyl acetate fraction,
AA- Ascorbic acid

DISCUSSION

In the DPPH free radical scavenging capacity assay by TLC, the EE, AE, EAF, MF, BF produced yellow or white spots in the purple background were considered as antioxidants.

There are numerous antioxidant methods for evaluation of antioxidant activity. For *in vitro* antioxidant screening, DPPH, nitric oxide, hydrogen peroxide and reducing power assay are most commonly used. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecule and radical progresses, results in the scavenging of the radical by hydrogen donation[18]. It is visually noticeable as a discolouration from purple to yellow. Figure 1 indicates noticeable effect of extracts and fractions on scavenging of free radicals.

The compound, SNP is known to decompose in aqueous solution at physiological pH (7.2) producing $NO^{\cdot}$. Under aerobic conditions $NO^{\cdot}$ reacts with oxygen to produce stable products (nitrate and nitrite). This leads to reduction of nitrite concentration in the assay media[19]. Here the extracts and fractions of *Cicer arietinum* pod wall exhibited potent nitric oxide scavenging activity.

H_2O_2 is highly important because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells[18]. The results showed that extracts and fractions of *Cicer arietinum* pod wall had an effective H_2O_2 scavenging activity.

Fe^{2+} transformation in the presence of EE and EAF was investigated and found to have significant reducing ability. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[18].

Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups and may contribute directly to antioxidative action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans[20]. Total amount of phenolic content present in *Cicer arietinum* shown in figure 5.

Total antioxidant capacity of EE and EAF was calculated based on the formation of phosphomolybdenum complex which was measured spectrophotometrically at 695nm.

In addition, the antioxidant activity may be due to phenolic compounds in *Cicer arietinum* pod extracts. However, the components responsible for the antioxidative activity of *Cicer arietinum* pod wall extracts are currently unclear. Therefore, it is suggested that further work be performed on the isolation and identification of the antioxidant components of *Cicer arietinum* pod wall.

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