# Comparative Analysis of Fractional Performance on Antioxidant Action of Red Gram (Cajanus Cajan) Seed Layer

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***Abstract – Red gram is a protein rich staple food. It contains about 22 percent protein and biological and medicinal properties not yet investigated particularly for red gram seed coat proteins. The present study evaluated the antioxidant activity of crude proteins in red gram seed coat for aqueous, ammonium sulphate precipitation and dialysis fraction. Antioxidant activity was assessed by DPPH and OH radical scavenging activity. The protein was varied between fractions range from 0.90-2.80mg, DPPH radical scavenging activity in fraction range found to be 36.16-61.20% and hydroxyl radical scavenging activity range from 32.2152.44%. Crude fraction (CF), ammonium sulphate fraction (AF) and Dialysis fraction (DF) assayed for antioxidant activity, among them DF activity is best as a protein concentrated. SDS-PAGE photograph showing six bands in the DF with low and high molecular weight proteins and DF exhibited heat stable up to 50$^{o}$C compare to room temperature (RT) and antioxidant activity. DF proteins stability checked and inhibited best at 40$^{o}$C 58.12%, 48.16% inhibition compare to RT 61.25%, 52.42%for DPPH and OH radical scavenging activity respectively. The key study indicated that these activities in crude fraction could contribute significantly to the pharmacological properties. These results suggest that the further purification and characterizations of protein is need and it may possess best biological and medicinal value.***

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## INTRODUCTION

The proteins introduce in the seeds of plants with the capacity to tie and agglutinate cells were distinguished throughout the most recent century and such proteins were called phytohemagglutinins in view of their capability to agglutinate red blood cells (Lis and Sharon, 1986). Proteins are moderately solvent and extraction is normally done by diverse routines incorporating dispersion in aqueous result ammonium and acetone precipitation. Plants have huge capacity to synthesize aromatic substances, the vast majority of which incorporate phenolic or their oxygen-substituted subordinates. It embodies auxiliary metabolites which help in plant guarding instruments that offer assurance against creepy crawlies, herbivores and microorganisms (Rios and Recio, 2005).there are numerous critical motivations to screen for novel elective antioxidant and antimicrobial substances from characteristic sources principally plants (Ankri and Mirelman, 1999). The lethality and reactions of the pills without further ado utilized as a part of human services and medicine being a real zone of concern. The era of pills in bounty from regular sources with additional viability, minimal effort of preparation and low or irrelevant symptoms has turned into a prime center of the pharmacological industry (Newman and Cragg, 2007). These days, there is a wide investment in the impacts of preparing on the antioxidant compounds of vegetables. Indeed, numerous bioactive compounds with antioxidant movement were available in vegetable seed. The bioactive compounds exhibit in the red gram seed might be separated into non-protein and protein compounds. Biotic ally animated peptides, proteins and phenolic compounds are either regularly handled by enzymatic absorption, aging, germination or enzymatic hydrolysis. As of late, there is a developing investment to recognize and use against oxidative compounds in numerous regular sources, for example, soy protein (Moure et al, 2000). Disregarding the physiological imperativeness, the universal Chinese medicine hypothesis accepts that dark soybean has been utilized as a segment as a part of antiquated medicines to treat diabetes, hypertension, hostile to maturing, cosmetology, blood course et cetera Cho et al. (2001) due to its animated peptide compounds.

Adipogenesis inhibitory peptide was isolated and identified from black soybean protein hydrolysate (Kim et al., 2007a).Exposure to various organic compounds including a number of environmental pollutants can cause cellular damage through metabolic activation of the compounds to highly free radical products. These free radical products induce lipid peroxidation, which is believed to be one of the major causes of cell membrane damage leading to a number of pathological events. Oxidation is a vital process in organisms and food stuffs. Oxidative metabolism is essential for cell survival but produces free radicals and other reactive oxygen species (ROS) which can cause oxidative changes. An excess of free radicals can overwhelm protective enzymes such as superoxide dismutase, catalase and peroxidase, causing destruction and lethal cellular effects (e.g., apoptosis) through oxidization of membrane lipids, cellular proteins, DNA, and enzymes which shut down cellular processes (Haque et al., 2009). Synthetic antioxidants such as butylatedhydroxyanisole (BHA)

and butylatedhydroxytoluene (BHT) are used as food additives and preservatives. Antioxidant activity in these synthetic antioxidants is stronger than that found in natural compounds such as a-tocopherol and ascorbic acid, but they are strictly regulated due to their potential health hazards, like carcinogenic potential and liver damage (Gulginet al, 2007; Wichi, 1988). Interest in the development and use of natural antioxidants as an alternative to synthetics has grown steadily; for instance, hydrolyzed proteins from many animal and plant sources have recently been found to exhibit antioxidant activity (Lee et al., 2010).The new approach to finding protective molecules that provide maximum protection of body organs with easy availability and minimal side effects is going on throughout the world. Many researchers report that proteins isolated from plant sources such as Curcuma comosarhizomes (Boonmee at al., 2011), Cicerarietiumseeds (Zhang et al., 2011; Li Y et al., 2008), Cajanusindicusleaves (Sarkar et al., 2006), wheat germ (Zhu et al., 2006) and Ginkgobilobaseeds (Huang et al., 2009) show antioxidant activity in vitro by DPPH assay.

Recently there has been a growing interest in the search for natural antioxidants for three principal reasons (Dastmalchietal., 2007): (i) numerous clinical and epidemiological studies have demonstrated that consumption of fruits and vegetables is associated with reduced risks of developing chronic diseases such as cancer, cardiovascular disorders and diabetes; (ii) safety considerations regarding the potential harmful effects of the chronic consumption of synthetic antioxidants in foods and beverages; and (iii) the public's perception that natural and dietary antioxidants are safer than synthetic analogues. PROTEINS are valued by the food manufacturer for their functional properties (emulsification, gelation, foaming etc.) and for their nutritional value. Of late there has been concern over the sustainability of some food protein sources such as fish meal protein, and the rising cost of others such as egg proteins and soy proteins. This has led to the investigation of alternative protein sources for food use which can be used to either partially or fully replace more expensive proteins. There is also an advantage in using material that was previously considered to be waste to recover useful functional proteins. Such waste includes the peel, skin and seeds of fruits, seed coat of legumes and vegetables, materials which are either discarded or used in low value commodities such as animal feed. Even research on soybean seed coat protein was done and identified "Bowman-Birk inhibitor (BBI)", possessing a molecular weight (MW) of 8 kD, is a known cancer chemo preventative and ant carcinogenic agent is identified by David et al., (2001).One waste product we are investigating to see fractions having useful protein antioxidant activity or not. There is limited published work focused on red gram seed coat even though they contain potentially useful quantities of protein. The objective of the present attempted study is to see the fraction and technique efficiency on antioxidant potency of red gram seed coat protein using simple aqueous extraction, ammonium sulphate precipitation and dialysis fraction.

## MATERIALS AND METHODS

Plant Material : The red gram seeds were gathered from the nearby market Bangalore, Karnataka. The seed were let in water overnight and seed layer was evacuated, shade dried for 48 h at room temperature. The dried examples were ground to a fine powder and utilized for extraction.

Chemicals: Bovine Serum Albumin (BSA), 2, 2, Diphenyl-1-picrylhydrazyl (DPPH), Folinciocalteu's phenol reagent, Methanol, Ferrous sulphate was bought from Sigma Chemicals (St. Louis, USA). Ammonium sulphate, Sodium dodecyl sulphate, Di-sodium hydrogen phosphate, n-hexane was bought from Merck. Deoxy ribose, Ammonium persulphate, TEMED was obtained from Sisco chemicals labs. BHA, Thibarbutryic harsh corrosive from Sigma and acidic harsh corrosive was obtained from Fischer logical. All different chemicals utilized were of expository evaluation.

Systems for extraction and fractionation of soluble antioxidant protein- Crude Fraction (CF): Fine powdered 2g example was disintegrated in 50ml of n-hexane upset 2h in revolving shaker and rehash the venture for two to three cycles to defatted the powder and dissolvable was vanished by utilizing blaze evaporator. The pellet was dried at room temperature until the dissolvable is vanished and

weighed. The dried seed layer powder was homogenized by utilizing 100ml of refined water.the suspension was hatched overnight at 4oc with consistent blending, then filtered and centrifuged at 13,000 rpm at 4oc for 20 min. The ensuing pellet was disposed of and supernatant carried to ammonium sulphate precipitation

Ammonium sulphate Fractionation (AF): According to the system depicted by Wang et al., (2003). The prepared crude concentrate was subjected to ammonium sulphate precipitation. In this step, pellet was structured after hatching for 4 h at 4 °c. The pellet was gathered by centrifugation at 3,000 rpm for 10 min at 0 °c was air dried and re-separated with frosty 10 % TCA and centrifuged at 3,000rpm for 10min. Protein deposit was gathered, washed until harsh corrosive free and after that air dried specimen utilized for protein estimation.

Dialysis Fraction (DF): The ammonium sulphate hastened example was disintegrated in suitable measure of twofold refined water. The example holding salts was evacuated by far reaching dialysis at 4oc with 6 progressions for three days against twofold refined water (ph= 7.4) at 2.5kda sub-atomic cutoff. The complete evacuation of salts was affirmed by checking with Nessler's test by 1:1 degree of specimen and Nessler's reagent as per Meloan and Kiser, (1963).

Gel electrophoresis - SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method Laemmli (1970). Sample for analysis were dissolved in sample buffer, boiled for 3min and centrifuged. The supernatant 42 ^g protein sample was loaded on to the gel. Electrophoresis was performed in a GeNei gel electrophoresis unit, with 12% separating (100v) and 3% (50v) stacking gels. Electrophoresis was performed at room temperature with 100 V until bromophenol blue tracking dye reached the bottom of the running gel. Molecular weight markers used were Lysozyme (14.3 kDa), Soyabeantrypsin inhibitor (20.1 kDa), Carbonic anhydrase (29.0 kDa), Ovalbumin (43.0 kDa), Bovine Serum Albumin (66.0 kDa) and Phosparylase b (97.4 kDa).The gels were stained overnight with Coomassie Brilliant Blue G-250 according to the method of Fling and Gregerson (1986); stained gels were destained with a mixture of 25% ethanol, 6% acetic acid and make the final volume to 100mL by using distilled water; destained gels were photographed.

DPPH Free radical scavenging activity-The scavenging activity of DPPH free radicals by different plant extracts was determined according to the method reported by Gyamfiet al., (1999) with slight modification. Extracted sample was mixed with 1 ml of 0.1 mM DPPH in methanol solution and 450 pl of 50 mMTris-HCl buffer (pH 7.4). Methanol (50 pl) only was used as the experimental control. After 30 min of incubation at room temperature, the reduction in the number of DPPH free radicals was measured, reading the absorbance at 517nm. BHT was used as controls. The percent inhibition was calculated from the following equation:

$$\% \text{ Inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of test sample}]}{[\text{Absorbance of control}]} \times 100$$

Hydroxyl radical-scavenging activity - Hydroxyl radicals were generated by a Fenton reaction system and the scavenging capacity towards the hydroxyl radicals was measured by using a deoxyribose method (Halliwell et al. 1987) with minor modifications. The reaction mixture containing deoxyribose (2.8mM), FeCl3(100pM), EDTA(104pM), ascorbic acid(100pM) and H2O2(1mM) were mixed with various concentrations of extract in phosphate buffer (KH2PO4- K2HPO4) 20 mM, pH 7.4 in 1ml final volume. Incubation was carried out for 1 h at 37°C and the reaction stopped by addition of 1 ml 1% (w/v) thiobarbituric acid (1gm in boiling water & cool then add)and the mixture was boiled for 20 min, cooled and add 1 ml acetone. Measured the absorbance at 535nm spectrophotometrically. BHT was used as positive control. Phosphate buffer, 20mM, pH 7.4 was used as a blankwas used as blank and the sample solution without deoxyribose as sample blank. The inhibition ratio was calculated from the following equation. The percent hydroxyl radical scavenging activity of extracts was determined accordingly in comparison with the negative control. Scavenging activity (%) = (A0 - (A1- A2)/A0) x 100. Where, A0, A1, and A2 are the absorbance's of the blank, extract (or BHT) and the sample blank, respectively at 532 nm.

## RESULTS

Yield and soluble protein in Cajanuscajan seed coat - Soluble proteins were isolated from dry red gram seed coat by aqueous extraction, followed by ammonium sulphate precipitation and dialysis. The ammonium sulphate precipitate was extracted with TCA in order to remove polysaccharides, phenolic compounds and the residue was collected by centrifugation. The yield and protein, so obtained varied between the fractions. It highlights the importance of methods and technique (Table-1).

| Treatments | Protein (mg) | Yield (%) | DPPH radical scavenging Activity (%) | OH radical scavenging Activity (%) |
|---|---|---|---|---|
| CF | 2.80±0.01 | 100±0.00 | 36.16±0.01 | 32.21±0.04 |
| AF | 1.41±0.01 | 47.19±0.01 | 48.35±0.02 | 42.26±0.02 |
| DF | 0.90±0.01 | 26.14±0.01 | 61.20±0.01 | 52.44±0.02 |

Table 1 Amount of protein and antioxidant activity in Cajanuscajanseed coat fractions.

Antioxidant activity - The antioxidant activities of the fractions were determined by measuring the DPPH free radical scavenging activity and hydroxyl radical-scavenging activity. DPPH is a lipophile radical that could be reduced by donation of either hydrogen or electrons. Table 1 expresses the effect of fraction protein scavenging activity. DPPH activity in CF, AF and DF, showed 36.16, 48.35 and 61.20 % inhibition respectively.

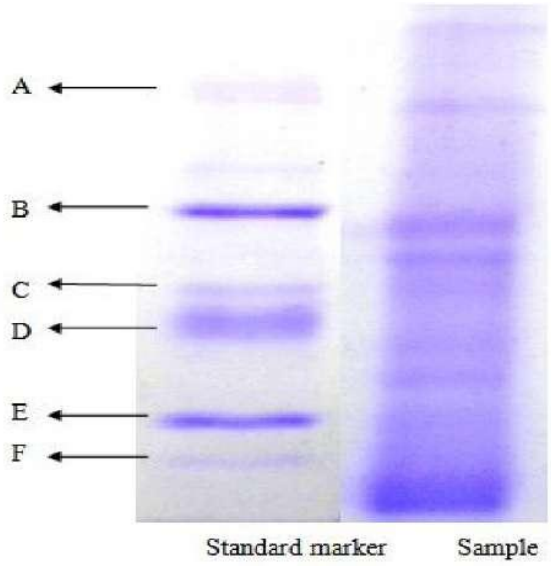


Figure 1. SDS Polyacrylamide Gel Electrophoresis of Cajanuscajan seed coat protein. Standard markerA=97.4 kDa (Phosparylase b), B=66.0 kDa (BSA), C=43.0 kDa (Ovalbumin), D=29.0 kDa (Carbonic anhydrase), E=20.1 kDa (Soyabean trypsin inhibitor), F=14.3 kDa (Lysozyme) used to compare the sample lane.

This means extract was observed to scavenge hydroxyl radical more in dialyzed fraction than other. The OH radical is the most toxic ROS as it can damage almost all vital macromolecules. BHT, used as positive control, was highly effective in quenching the OH radical. The results obtained 32.12, 42.26 and 52.44% in CF, AF and DF respectively. Several studies used the deoxyribose system to assess the biological activity of various natural plant- derived biomolecules and reported that molecules which are able to chelate iron might have scavenging ability on OH radicals. In our investigation also, all fractioned extracts showed a strong ability to quench DPPH and OH radicals effectively (Table-1).

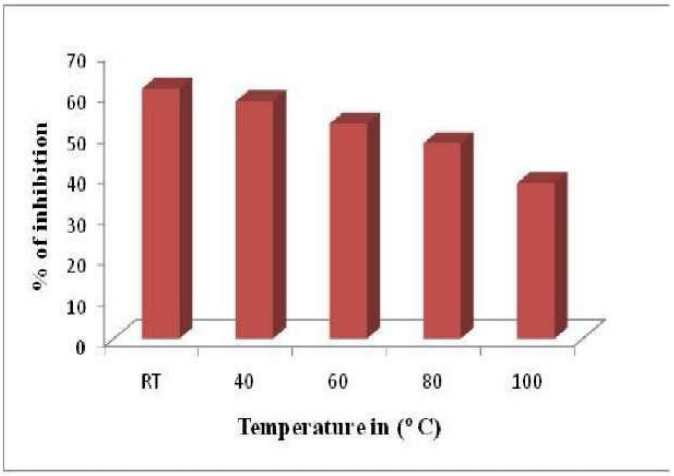


Figure 2 Heat stability DPPH antioxidant activity at different temperatures.

SDS-PAGE - To separate the size of the protein, SDS-PAGE was performed using 12% polyacrylamide as the resolving gel and 3% polyacrylamide as the stacking gel and the bands were stained by Coomassie Brilliant Blue G-250 staining method. Then the bands were visualized by gel documentation system and the standard markers used range from 14.3-97.4 kDa molecular weights A-F and the crude dialyzed sample protein was run along with the standard, showing about six bands. Photograph highlighting compare to standard lane, presence of very low and high molecular weight proteins in the sample lane (Figure 1).

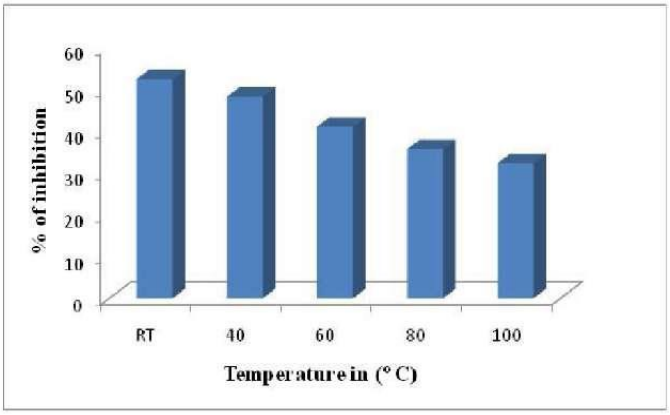


**Figure 3 Heat stability OH radical antioxidant activity at different temperatures.**

Effect of temperature on antioxidant activity : The presence of proteins confirmed by gel electrophoresis and stability of the proteins to heat and its activity was measured at different temperatures for 10 minutes for DF. The different temperature treatment protein was assessed for DPPH free radical scavenging activity as well as hydroxyl radical-scavenging activity. Protein activities higher in DPPH compare to the OH radical scavenging activity, at RT 61.25%, 52.42% and at 40°C 58.12%, 48.16% inhibition is obtained respectively. It suggest that stable highly up to 50°C but at higher temperatures crude protein indicating that the antioxidant activity was heat unstable slightly at higher temperature (Fig 2 & 3).

## DISCUSSION

In this paper studycarried out for Cajanuscajanseed coatandfocused on fractionations and its antioxidant movement in the aqueous medium to see the proficiency of procedure. The bioactive proteins incorporate an antifungal protein from C. longa (Wang and Ng, 2005; Petnual et al., 2010), haemagglutinating proteins (lectins) from C. amarissima (Kheeree et al., 2010), C. aromaticaandc.zedoaria (Tipthara et al., 2007) and antioxidant compound from C. Comosa(boonmee et al., 2011). The mannose tying lectin showing haemmaglutinating movement disconnected from rhizomes of C. zedoariawas found to compare to an atomic mass of 13 kda (Tipthara et al., 2007). The present studies indicated that soluble proteins were available in red gram seed cover well, with yields from 47.20-26.12% in the AF and DF division individually. Soybean seed layer protein "Bowman-Birk inhibitor (Bbi)", is a known malignancy chemopreventative and anticarcinogenic operator is recognized by David et al., (2001). Red gram seed cover crude proteins indicated critical antioxidant action, (measured as far as DPPH free radical rummaging movement and hydroxyl radical-searching action) which was stable to high temperature at 40°c contrast with RT however somewhat temperamental at higher temperature.

The soluble and high temperature security of the proteins display in aqueous concentrates of distinctive parts of red gramcould further improve their pharmacological activity in examination with other bioactive phytochemicals, for example, phenolics, crucial oils and flavonoids show in the seed. With this foundation the study demonstrated that, red gram soluble crude protein parts had powerful antioxidant movement property. As per (Abbiw, 1990; Prema & Kurup, 1973) the red gram seed concentrates demonstrated living and medicinal lands yet seed cover proteins are not yet built. The advanced manufactured BHT and BHA demonstrating antioxidant and in addition antimicrobial movement. Separated from that these compounds are high cost and unfriendly symptoms on the host. With these foundation scientists persistently improving such kind of compounds for novel contaminations and the microorganisms advancing safety make recently existing anti-toxins less successful and reactions. In such situations, characteristic items which are a piece of our every day eating methodology serve as the best applicants for new antioxidant and antimicrobial pill revelation. These impacts can overcome by utilizing nourishment grade intense antioxidants with no reactions and cost viably. In these paper crude protein portions antioxidant action spoke to by SDS-PAGE groups proteins show in the specimen. Further decontamination, ID and characterization of protein are done in next study.

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