RESEARCH ARTICLE

COMPARATIVE EVALUATION OF FRACTIONAL EFFICIENCY ON ANTIOXIDANT ACTIVITY OF RED GRAM (Cajanus cajan) SEED COAT CRUDE PROTEIN EXTRACTS

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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 32.21-52.44%. 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°C compare to room temperature (RT) and antioxidant activity. DF proteins stability checked and inhibited best at 40°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 characterization of protein is need and it may possess best biological and medicinal value.

INTRODUCTION

The proteins present in the seeds of plants with the ability to bind and agglutinate cells were identified during the last century and such proteins were called phytohemagglutinins because of their ability to agglutinate red blood cells (Lis and Sharon, 1986). Proteins are relatively soluble and extraction is usually carried out by different methods including diffusion in aqueous solution ammonium and acetone precipitation. Plants have enormous ability to synthesize aromatic substances, most of which include phenolics or their oxygen-substituted derivatives. It comprises of secondary metabolites which help in plant defensive mechanisms that offer protection against insects, herbivores and microorganisms (Rios and Recio, 2005). There are many important reasons to screen for novel alternative antioxidant and antimicrobial substances from natural sources mainly plants (Ankri and Mirelman, 1999). The toxicity and side effects of the drugs presently used in health care and medicine being a major area of concern. The generation of drugs in plenty from natural sources with more efficacy, low cost of production and low or negligible side effects has become a prime focus of the pharmacological industry (Newman and Cragg, 2007). Nowadays, there is a wide interest in the effects of processing on the antioxidant compounds of legumes. Indeed, many bioactive compounds with antioxidant activity were present in legume seed. The bioactive compounds present in the red gram seed can be divided into non-protein and protein compounds. Biologically active peptides, proteins and phenolic compounds are either naturally produced by enzymatic digestion, fermentation, germination or enzymatic hydrolysis. In recent years, there is a growing interest to identify and utilize anti-oxidative compounds in many natural sources, such as soy protein (Moure et al., 2000).

In spite of the physiological importance, the traditional Chinese medicine theory believes that black soybean has been used as a component in ancient medicines to treat diabetes, hypertension, anti-aging, cosmetology, blood circulation and so on Cho et al. (2001) because of its active 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 oxidation of membrane lipids, cellular proteins, DNA, and enzymes which shut down cellular processes (Haque et al., 2009). Synthetic antioxidants such as butylated hydroxyanisole (BHA)
and butylated hydroxytoluene (BHT) are used as food additives and preservatives. Antioxidant activity in these synthetic antioxidants is stronger than that found in natural compounds such as α-tocopherol and ascorbic acid, but they are strictly regulated due to their potential health hazards, like carcinogenic potential and liver damage (Gülçin et 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 *Cucuruma comosatizomes* (Boonmee et al., 2011), *Cicerarietium* seeds (Zhang et al., 2011; Li Y et al., 2008), *Cajanusindicus* leaves (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 kDa, is a known cancer chemopreventative and anticarcinogenic agent 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 collected from the local market Bangalore, Karnataka. The seed were soaked in water overnight and seed coat was removed, shade dried for 48 h at room temperature. The dried samples were ground to a fine powder and used for extraction.
Determination of total crude protein

The protein estimation was determined by Bradford’s method (Bradford, 1976) using bovine serum albumin as a standard.

Antioxidant activity

Antioxidant activity was determined by measuring DPPH free radical scavenging activity and Hydroxyl radical-scavenging activity.

DPPH Free radical scavenging activity

The scavenging activity of DPPH free radicals by different plant extracts was determined according to the method reported by G Yamfiet al., (1999) with slight modifications. Extracted sample was mixed with 1 ml of 0.1 mM DPPH in methanol solution and 450 µl of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 µl) 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 517 nm. BHT was used as controls. The percent inhibition was calculated from the following equation:

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\% \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(100µM), EDTA(104µM), ascorbic acid(100µM) 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 aceton. Measured the absorbance at 535nm spectrophotometrically. BHT was used as positive control. Phosphate buffer, 20mM, pH 7.4 was used as a blank, A was 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.

Effect of temperature on antioxidant activity

The protein solutions were heated in a water bath at temperatures ranging from 40, 60, 80 and 100°C for 10 min, cooled to room temperature and assayed for protein, antioxidant activity by DPPH free radical scavenging assay and Hydroxyl (OH) radical–scavenging activity.

Statistical Analyses

Assays were performed in triplicate, and the results were expressed as mean values with standard deviations (SD).

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).

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

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Protein (mg)</th>
<th>Yield (%)</th>
<th>DPPH radical scavenging Activity (%)</th>
<th>OH radical scavenging Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>2.80±0.01</td>
<td>100±0.00</td>
<td>36.16±0.01</td>
<td>32.12±0.04</td>
</tr>
<tr>
<td>AF</td>
<td>1.41±0.01</td>
<td>47.19±0.01</td>
<td>48.35±0.02</td>
<td>42.26±0.02</td>
</tr>
<tr>
<td>DF</td>
<td>0.90±0.01</td>
<td>26.14±0.01</td>
<td>61.20±0.01</td>
<td>52.44±0.02</td>
</tr>
</tbody>
</table>

Values are the mean of triplicate analysis ± standard deviation.

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 (Phosphorylase b), B=66.0 kDa (BSA), C=43.0 kDa (Ovalbumin), D=29.0 kDa (Carbonic anhydrase), E=20.1 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).
proteins include an antifungal protein from *C. longa* (Wang and Ng, 2005; Petual et al., 2010), haemagglutinating proteins (lectins) from *C. amarissima* (Kheeree et al., 2010), *C. aromaticum* and *C. zedoaria* (Tipthara et al., 2007) and antioxidant enzyme from *C. Comosa* (Boonmee et al., 2011). The mannose binding lectin exhibiting haemagglutinating activity isolated from rhizomes of *C. zedoaria* was found to correspond to a molecular mass of 13 kDa (Tipthara et al., 2007). The present studies showed that soluble proteins were present in red gram seed coat well, with yields from 47.20-26.12% in the AF and DF fraction respectively. Soybean seed coat protein “Bowman-Birk inhibitor (BBI),” is a known cancer chemopreventative and anticarcinogenic agent is identified by David et al., (2001). Red gram seed coat crude proteins showed significant antioxidant activity, (measured in terms of DPPH free radical scavenging activity and hydroxyl radical scavenging activity) which was stable to heat at 40°C compare to RT but slightly unstable at higher temperature.

The soluble and heat stability of the proteins present in aqueous extracts of different fractions of red gram could further enhance their pharmacological action in comparison with other bioactive phytochemicals such as phenolics, essential oils and flavonoids present in the seed. With this background the study showed that, red gram soluble crude protein fractions had potent antioxidant activity property. According to (Abbiw, 1990; Prema & Kurup, 1973) the red gram seed extracts showed biological and medicinal properties but seed coat proteins are not yet established. The developed synthetic BHT and BHA showing antioxidant as well as antimicrobial activity. Apart from that these compounds are high cost and adverse side effects on the host. With these background researchers continuously developing such type of compounds for novel infections and the microorganisms developing resistance make already existing antibiotics less effective and side effects. In such scenarios, natural products which are a part of our daily diet serve as the best candidates for new antioxidant and antimicrobial drug discovery. These effects can overcome by using food grade potent antioxidants with no side effects and cost effectively. In these paper crude protein fractions antioxidant activity represented by SDS-PAGE bands proteins present in the sample. Further purification, identification and characterization of protein are carried out in next study.

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**Conflict Of Interest**

There is no conflict of interest associated with the authors of this paper.

**References**


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**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).

**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 compared to the OH 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 studied carried out for *Cajanus cajan* seed coat and focused on fractionations and its antioxidant activity in the aqueous medium to see the efficiency of technique. The bioactive proteins include an antifungal protein from *C. longa* (Wang and...


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