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Identification of metabolites from an active fraction of *Cajanus cajan* seeds by high resolution mass spectrometry

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Metanephrin (PubChem CID: 21100)
Tyramine (PubChem CID: 5610)
2-Methylene-5-(2,5-dioxotetrahydrofuran-3-yl)-6-oxo-10,10-dimethylbicyclo[7:2:0]undecane (PubChem CID: not available)
Benazepril (PubChem CID: 5362124)
Prometone (PubChem CID: 4928)

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ABSTRACT

Antioxidants are important food additives which prolong food storage due to their protective effects against oxidative degradation of foods by free radicals. However, the synthetic antioxidants show toxic properties. Alternative economical and eco-friendly approach is screening of plant extract for natural antioxidants. Plant phenolics are potent antioxidants. Hence, in present study *Cajanus cajan* seeds were analyzed for antioxidant activity, Iron chelating activity and total phenolic content. The antioxidant activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay showed 71.3% inhibition and 65.8% Iron chelating activity. Total 37 compounds including some short peptides and five major abundant compounds were identified in active fraction of *C. cajan* seeds. This study concludes that *C. cajan* seeds are good source of antioxidants and Iron chelating activity. Metabolites found in *C. cajan* seeds which remove reactive oxygen species (ROS), may help to alleviate oxidative stress associated dreaded health problem like cancer and cardiovascular diseases.

1. Introduction

In tropical and semi-tropical world *Cajanus cajan* is a major non toxic, edible herb, grain legume crop (Duke, 1981). It is grown in about 50 countries in Asia, Africa and America for multiple uses like food and fodder. Around 76% of the total global area and around 73% of total global production of *C. cajan* falls in India (Ali, 1990). Plants are the main source of secondary metabolites which help them to fight against environmental stress or pest attack. Secondary metabolites show various therapeutic uses in medicine from time immemorial (Pal, Sarkar, Gain, Jana, & Mandal, 2011).

Phenolic compounds are secondary metabolites of plants which constitute most common and widespread groups. Several thousand compounds are identified with a large range of structure: monomeric, dimeric and polymeric phenolics among which over 8150 are flavonoids (Datta & Lal, 2012). Phenolic compounds have been found to be one of the best active antioxidant ingredients in plant foods, including grains, vegetables and fruits (Imaga et al., 2009). The use of synthetic antioxidant in food product is discouraged due to their carcinogenic potential (Chatha, Hussain, Bajwa, Sherazi, & Shaukat, 2011). A natural source like herbs contained hundreds of compounds with promising antioxidant activity (Ei & Karakaya, 2004). Natural antioxidants could lower the risk of cardiovascular disease and several cancers (Jiang, Zhan, Liua, & Jiang, 2008). The major active compounds in *C. cajan* are flavonoids, stilbenes, coumarins possess anti-inflammatory, anti-microbial, antioxidant, antitumor and antiviral activity (Wu et al., 2009, 2011). However, in India *C. cajan* is a most important pulse crop where mainly seeds are consumed. As per our knowledge, no reports are available for characterization of compounds from

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; *C. cajan*, *Cajanus cajan*; ESI-Q-TOF-MS, Electrospray ionization Quadrupole Time of Flight mass spectrometry; LC/TOF-MS, liquid chromatography time of flight mass spectrometry; ND, not detected; GAE, gallic acid equivalents; GC–MS, gas chromatography mass spectrometry.

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C. cajan seeds. Hence present study is undertaken to characterize such type of compounds from *C. cajan* seeds.

Various analytical methods are employed for the separation and identification of metabolites which include high performance liquid chromatography – ultra violet detector (Kong et al., 2009). These methods are insufficient due to their limitation regarding sensitivity, resolution etc. Ultra high performance liquid chromatography has been preferred due to less solvent consumption and reduction in run time (Narayan & Kumar, 2013). LC/TOF-MS analysis gives accurate mass measurements, high resolution and provides the elemental compositions of unknown peaks with more accuracy in complex matrices (Ferrer, Garcıa-Reyes, Mezcua, Thurman, & Fernandez-Alba, 2005; Segura-Carretero et al., 2008).

The objective of the current study was DPPH radical scavenging and Iron chelating activity guided fractionation by a column chromatography and identification of metabolites by ESI-Q-TOF-MS from *C. cajan* seeds.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals were procured from different reputed companies mentioned in bracket, DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma Aldrich), gallic acid, Folin–Ciocalteau (SRL), sodium carbonate (Heramb laboratories), HPLC-grade methanol, ethyl acetate, hexane, chloroform, ethanol, acetone. (Rankem), O-phenanthroline (Qualigens), ferric chloride (Sarabhai M. chemicals) and L-ascorbic acid (Polypharma). All chemicals and reagents used in this study were of analytical grade.

2.2. Plant material

The mature seeds (Variety BDN 711) of *C. cajan* were collected from Dabhadi, Taluka Badnapur, District Jalna, Maharashtra, India in January 2015. Seeds were thoroughly washed with distilled water and dried at room temperature. Completely dried seeds were crushed into fine powder (2 kg) by grinder mixer.

2.3. Extraction and fractionation

Fine dried powder (2 kg) was sequentially soaked in non-polar to polar solvents (1:6 w/v) for preliminary fractionation (Fig. 1). Fractionation was carried out at room temperature (around 27 °C) for 10–12 h with intermittent shaking of extraction flask and finally filtered through ordinary filter paper. Resulting extracts were *n*-hexane, chloroform, ethyl acetate and methanol. Out of these four extracts ethyl acetate and methanol extracts exhibited DPPH radical scavenging activity. The methanol extract showed the highest activity. Hence methanol from extract was evaporated to dryness and the resulting weight of extract was 43 g (sticky and brown color). This extract was subjected for solid-liquid fractionation by using increasing order of linear gradient of methanol in chloroform [2:8 (**A**), 4:6 (**B**), 6:4 (**C**), 8:2 (**D**) and 10:0 (**E**) v/v]. Fraction **(B**) exhibited prominent DPPH radical scavenging activity as compared to other fractions. Therefore, fraction (**B)** 6 g was further fractionated by using silica gel (60–120 mesh) column chromatography by applying a linear gradient of methanol in chloroform. Total 10 fractions (F1, F2…F10) were collected and the volume of each fraction was 200 ml. Fraction 7 exhibited more DPPH radical scavenging activity than other fractions. Thus, fraction 7 was selected for analysis by ESI-Q-TOF-MS for identification of metabolites.

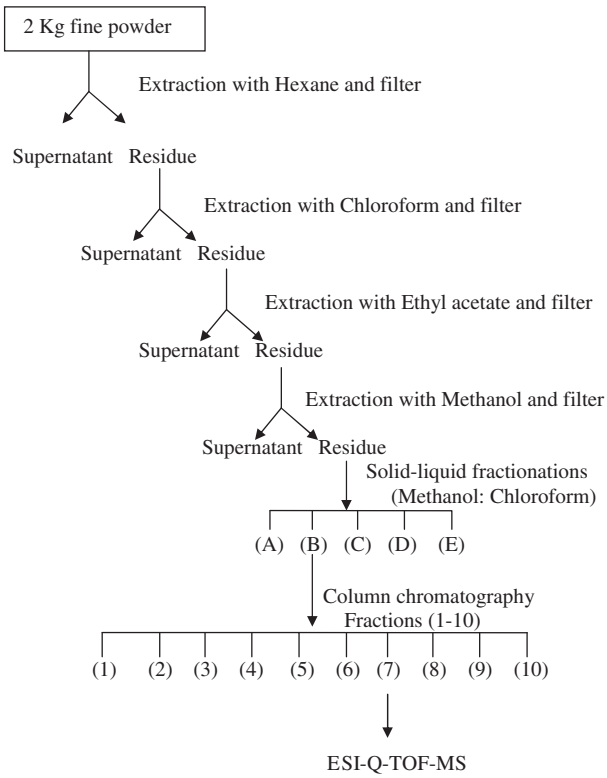

Fig. 1. Schematic representation of the DPPH activity guided fractionation and identification of the metabolites from *C. cajan* seeds.

2.4. Total phenolic content

Amount of total phenolic in all fractions were determined by using Folin-Ciocalteau assay with slight modification of the previously reported method (Ainsworth & Gillespie, 2007). Gallic acid was used as a reference standard for plotting of calibration curve. Fixed volume (40 µl) of each fraction was diluted up to 1.5 ml with de-ionized water. This was mixed with 0.5 ml Folin-Ciocalteau reagent and incubated at room temperature for 3 min. Thereafter, each aliquot was neutralized with 1 ml sodium carbonate (7% w/v). Reaction mixtures were incubated at room temperature for few min with intermittent shaking for color development. Absorbance of resulting blue color was measured at 650 nm using double beam UV–vis spectrophotometer. Amount of total phenolic was estimated from standard graph of gallic acid and expressed as gallic acid equivalents µg GAE/mg of extract.

2.5. Scavenging activity by DPPH assay

DPPH radical scavenging activities of all fractions were carried out by using a slight modification of the reported method (Yamaguchi, Takamura, Matoba, & Terao, 1998). For a usual reaction, 2 ml of 100 µM DPPH in ethanol/acetone was mixed with various concentrations of extract (40, 80, 120…200 µg/ml). The ascorbic acid was used as standard reference antioxidant. All reaction mixtures were kept in the dark for 15 min and the optical density was noted at 517 nm. For the control, DPPH in ethanol/acetone was taken without extract and the optical density was recorded after 15 min. Each assay was carried out in triplicate. The decrease in O.D. of DPPH by the addition of test samples in relative to the control was used to calculate the antioxidant activity. The antioxidant activity of each sample was calculated using the following Eq. (1):