2.1 INTRODUCTION:

Phytochemicals are chemical compounds formed during the normal metabolic process in plants. They usually occur in complex mixtures that differ among plant organs and stages of development. Higher plants are warehouses of phytochemicals which are useful in the pharmaceutical industry. Some beneficial pharmaceutical actions of plant materials results from the combination of secondary metabolic products that are present in the plant (Lee et al., 2003; Pietta, 2000). Although secondary metabolic products may have a variety of functions in plants, it is likely that their ecological function may have some bearing on potential medicinal effects in humans.

The 20th century brought further understanding of human health the development of synthetic or semi synthetic analogs of plant compounds that led to drugs with higher level of potency over the past decade there has been an increased interest in phytochemicals for the purpose of human health and other benefits in the food industry.

Phytochemical screening of diet plants is very important in identifying sources of therapeutically and industrially important compounds. It is imperative to initiate an urgent step for screening of plants for secondary metabolites. The present work is an attempt to assess the status of phytochemical properties of C. cajan to improve the health status of people and also to use in pharmaceutical products of commercial
importance. This work therefore is designed to phytochemically screen
*C. cajan* leaf, seed coat and cotyledon with the objective of observing and
analyzing their respective chemical constituents.

2.2 REVIEW OF EARLIER WORKS:

A perusal of literature has shown the voluminous phytochemical
aspects of the legumes an attempt is made here to highlight the work briefly.

Available literature reviews that pigeon pea is capable to prevent and
cure a number of human ailments such as bronchitis, coughs, pneumonia,
respiratory infections, dysentery, menstrual disorders, sores, wounds,
abdominal tumours, tooth ache and diabetes (Kul Bhushan Saxena *et al*., 2010).

Jagroop (1991) conducted studies on gene expression in response to pigeon
pea root exudates and also studied spectral analysis using ultraviolet absorption,
infrared spectra, proton nuclear magnetic resonance and mass spectrometry.

Singh (1993) has reported the protein quality of pigeon pea (*C. cajan*)
as influenced by seed polyphenol. True protein digestibility (TD), biological
value (BV) and protein utilization (NPU) of low-polyphenol pigeon pea
cutivars (Nylon, BDN 2 and ICPL 87067) were significantly higher than
those of the high polyphenol cultivars (C 11, ICPL 87 and ICPL 151) when
whole-seed samples were compared. Most of the polyphenols (80-90%) were
concentrated in the seed coat. Dhal sample of low (Nylon) and high (C 11)
polyphenol cultivars revealed no large differences in TD, BV, and NPU
values of these cultivars. This indicated an adverse affect of seed coat
polyphenols on protein quality of pigeon pea whole-seed.
Agenieszka Troszynska and Ewa Cisk (2002) have compared the composition and contents of phenolic acids and condensed tannin in the seed coats of white and coloured varieties of pea (*Pisum sativum*).

Oliver Yu *et al.* (2003) have reported the isoflavone levels in *Glycine max* (soyabean). They showed the isoflavone levels were increased via metabolic engineering of the complex phenylpropanoid biosynthetic pathway.

In 2003, Soghra and Rizwan have characterized a proteinase inhibitor in seeds of *C. cajan*. Further, Chougle *et al.* (2004) have analysed the inhibitors such as proteinase, amylase, lectins and raffinose family oligosaccharides in mature seeds of different pigeon pea accessions (untreated) and compared with mutant lines.

Zohar *et al.* (2005) have investigated the microwave-assisted extraction (MAE) of bioactive saponins from chickpea (*Cicer arietinum* L). They suggest that potential using MAE for the efficient extraction of natural products may assist in expediting the chemical analysis and characterization of the biological activities of such compounds.

Yuan-gang Zu *et al.* (2006) have determined the four Flavonoids such as quercetin (QU), luteolin (LU), apigenin (AP) and isorhamnetin (IS) in pigeon pea leaf using RP-LC-DAD methods.

Yujie Fu *et al.* (2006) have evaluated the performance and separation characteristics of eight macroporous resins for the separation of luteolin (LU)
from pigeon pea leaves. They provide the scientific references for the large-scale LU production from pigeon pea or other plants extracts.

Bhattacharya and Chenchaiah (2007) have studied the seed coat phenolic compounds of *Cajanus cajan* as chemical barrier in formulation of artificial diet of *Spodoptera litura* (f.) larvae. The phenolic compounds were extracted from the seed coat of red gram and added in different proportions in the dehusked red gram based diet and similar studies also carried out with residue obtained after extraction of seed coat. It was observed that larvae of *S. liture* could not tolerate phenolic compounds at early stages while at later stages they might have tolerated the toxic effect or metabolized it to some either non-toxic compound. It was also proved that residue obtained after extraction of phenolic compounds also contained some substances responsible for the suppression of larval development. The study showed the presence of 18 different peaks but only four peaks were identified as chlorogenic acid, gallic acid, hydroquinones and syringic acid.

Ahalya et al. (2007) have investigated that husk of tur dal (*C. cajan*) as a new bio sorbent for the removal of Fe (III) and Cr (VI) ions from aqueous solutions. The results revealed that characterization of tur dal husk has an excellent material for treating waste water containing low concentration of metal ions.

Panighrahi et al. (2007) have reported the genomic relationships among 11 species in the genus *Cajanus* from two *Cajanus cajan* cultivars (DSLR-17 and
BDN-2) and ten wild species including *C. cajanifolius*, *C. lineatus*, *C. sericeus*, *C. acutifolius*, *C. lanceolatus*, *C. reticulates*, *C. albicans*, *C. scarabaeoides*, *C. volubilis* and *C. platycarpus*, as revealed by seed protein (albumin and globulin) polymorphisms resulted in 34 albumin and 27 globulin polypeptides by SDS-PAGE method. It has been hypothesized that cultivated pigeon pea has evolved through multi-genomic interaction involving *C. cajanifolius* and that it has experienced minor genomic reorganization during its divergence.

Subodh Gupta *et al.* (2009) have isolated the heterologous expression of legumin gene in *E. coli* from cDNA clones of immature seeds of pigeon pea (*C. cajan*). Legumin protein was extracted from pigeon pea seeds of different developmental stages (5th to 2nd) day after flowering (DAF) and characterized. The legumin gene (leg) of size 1.482 kb was screened, using the deoxyxygen in-labelled legumin probe, from the complementary deoxyribo nucleic acid (cDNA) library, constructed from 18-day-old (DAF) immature seeds of pigeon pea and sequenced (accession no. AF3555403). The legumin gene was sub-cloned in vector pET-24a driven by the bacterial promoter and its expression was detected in *Escherichia coli* by immunobloting using polyclonal antibodies, raised against the purified legumin protein.

Wu *et al.* (2009) have isolated four important compounds, pinostrobin, cajaninstilbene acid, vitexin and orientin from ethanolic extracts of leaves of *C. cajan*. The result shows that these compounds possess significant antioxidant properties.
Luo et al. (2010) have isolated cajanol an isoflovanone found in the roots of *C. cajan*. The result shows that cajanol a novel anticancer agent induces apoptosis in human breast cancer cells through a ROS mediated mitochondrial pathway.

Arawande and Borokini (2010) have studied the chemical composition and functional properties of two underutilized legume seeds (Jack beans and pigeon pea) flours were compared with that of the popularly consumed cowpea seed flour found in Nigeria. The result revealed that jack bean and pigeon pea seed flours are good functional foods for nutrition and utilization.

### 2.3 MATERIALS AND METHODS:

The experimental details of collection of plant materials, extract preparation and methods used for analyzing the phytochemicals are as follows:

#### 2.3.1 Collection of Plant Materials:

The leaves and seeds of *C. cajan* var. Maruti (ICP-8863) was collected from the field of Gulbarga District in Karnataka. The leaves were dried under shade and made into fine powder using pestle and mortar (Plate – 2.1). The seeds were moistened for 1 h and then dried in oven at 55°C overnight for separation of seed coat and cotyledon (dhal). The hull or seed coat was removed mechanically by means using hand grinder. The two fractions like seed coat and cotyledon were made into fine powder using grinder (Plate – 2.2).
Plate 2.1: Leaf Powder of *C. cajan*
Plate 2.2: a - Seeds of *C. cajan* var. Maruti (*ICP-8863*); b - Cotyledon (dhal);

c - Seed coat; d - Cotyledon powder and e - Seed coat powder
These powdered leaf, seed coat and cotyledons are used for extract preparation and quantitative phytochemical studies.

2.3.4 Quantitative Estimation of Primary Metabolites

The dried and powdered plant material of *C. cajan* viz., leaf, seed coat cotyledon were used for quantitative estimation of primary metabolites such as proteins, carbohydrates, lipids, DNA and RNA by using different methods are as follows.

2.3.4.1 Proteins:

Plant material of 0.5 g was homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.0) by grinding with a pestle and mortar. The homogenate was centrifuged at 15,000 g at 4⁰C for 15 min the supernatant thus obtained was used for protein estimation.

Protein estimation was made according to Lowry’s method (Lowry *et al.*, 1951). Plant extract (0.1 ml) is taken in test tubes and the volume was made up to 1 ml using distilled water. A 5 ml of the alkaline copper solution was added and incubated for 10 min then 0.5 ml of Folin-Ciocalteu’s reagent was added to the mixture and allowed to stand for 30 min and measure the absorbance at 660 nm against the reagent blank. Protein content was calculated using the calibration curve of bovine albumin.

2.3.4.2 Carbohydrates:

Total carbohydrate was estimated by Anthrone reagent (Yemm and Willis, 1954). Plant aliquot (0.05 ml) is taken in test tubes and the volumes was made up to 1 ml., to this solution 4 ml of Anthrone reagent was added
and mixture was heated in boiling water bath for 8 min followed by cooling. Optical density of green colour to dark colour was read at 630 nm.

2.3.4.3 Lipids:

Total lipid was estimated by the method of Barned and Blackstock (1973). The plant tissue was homogenized in 5 ml of chloroform and methanol mixture (2:1). The homogenate is filtered using Whatmann filter paper No. 1. Then, 0.1 ml of the filtrate is taken and left aside for evaporation. After complete evaporation, 1 ml of H$_2$SO$_4$ was added to the tube and boiled for 10 min to 0.2 ml of this solution 5 ml of vanillin reagent is added and shaken vigorously. The colour thus obtained is read at 520 nm, after 10 min against a reagent blank. The content of lipids was calculated using the calibration curve of cholesterol.

2.3.4.4 DNA and RNA:

A known mass of the plant tissue was homogenized with 10 ml ice-cold 10% tricloroacetic acid and further centrifuged at 5000 rpm for 10 min the precipitate obtained was treated with 5 ml of 1:1 ethanol : ether mixture and was again centrifuged at 5000 rpm. To the precipitate thus obtained a 5 ml of 0.5 N NaOH was added and incubated overnight at 37$^\circ$ C. The mixture was further centrifuged and thus supernatant thus obtained was subjected to the RNA estimation and the precipitate, to the DNA estimation.

The estimation of RNA was made by Orcinol method as described by Plummer (1985). The supernatant was treated with 2 ml of Orcinol reagent in a
boiling water bath. The mixture was cooled and optical density was measured at 665 nm content of RNA was estimated by using standard curve of RNA.

The estimation of DNA was made by Diphenylamine method as described by Plummer (1985). The precipitate was treated with 5 ml of Diphenylamine reagent and heated for 15 min in a boiling water bath. The mixture was cooled and the optical density was measured at 595 nm content of DNA was estimated by using standard curve of DNA.

2.3.2 Extract Preparation:

500 grams of the powdered leaf, seed coat and cotyledon were subjected to the Soxhlet successive extraction method (60-80 °C) using 2.5 lit. of pet ether, chloroform, ethanol and aqueous solvent for a period of 18 h. The extracts obtained were dried at 40 °C.

2.3.3 Preliminary Screening tests for Secondary Metabolites:

The preliminary tests for presence or absence of secondary metabolites were made by using successive crude extract viz., pet ether, chloroform, ethanol and aqueous extracts of leaf, seed coat and cotyledon of C. cajan were separately tested by the methods Harborne (1998); Gibbs (1974) and Sadasivam and Manickam (1992).

Tests for Phenols:

**Phenol test:** 0.5 ml of FeCl₃ (w/v) solution was added to 2 ml of test solution, formation of an intense colour indicates the presence of phenols.

**Ellagic acid test:** The test solution was treated with few drops of 5% (v/v) glacial acetic acid and 5% (w/v) NaNO₂ solution. The solution
turns muddy yellow, olive brown, Niger brown, deep chocolate colours depending on the amount of ellagic acid present.

Tests for Tannins:

**Gelatin test:** The test solution was evaporated to dryness and the resulted residue was dissolved in 1% (w/v) liquefied gelatine. To this, 10% (w/v) NaCl solution was added. A white precipitate was obtained which indicate the presence of tannins.

Tests for Flavonoids:

**Pew’s test:** A pinch of zinc powder and about 5 drops of 5 N HCl were added to the test solution. It results deep purple red (dihydroquercetin) or cherry red (dihydrokaempferol) colours. Flavonones, dehydrochalcons and other flavonoids get at most pinkish or brownish colour.

**Shinoda test:** A pinch of magnesium powder and 5 N HCl were added to the test solution and a deep red or magenta colour formation indicates the presence of flavanone or dihydroflavanol. However, dihydrocarchalcones and other flavonoids did not react with this reagent.

**NaOH test:** 1 ml of 1 N NaOH solution was added to the 1 ml of test solution, formation of yellow colour indicates the presence of flavonoids.

Tests for Lignins:

**Labat test:** formation of olive green colour, when the gallic acid is added to the test solution, indicates the presence of lignin.

**Lignin test:** formation of red colour, when 2% (w/v) furfuraldehyde is added to the test solution, indicates the presence of lignin.
Tests for Steroids:

**Libermann-Burchard test:** A green colour was formed, when the Libermann-Burchard reagent is added to the solution, indicate the presence of steroids.

**Salkowski’s test:** A wine red colour was developed when chloroform and Conc. H$_2$SO$_4$ were added to the test solution; indicate the presence of steroidal nucleus.

Tests for Alkaloids:

**Iodine test:** 1 ml of KI in iodine solution was added to the 2 ml of test solution. A brown precipitate formation indicated the presence of alkaloids.

**Dragendorff’s reagent:** 2 ml of Dragendorff’s reagent and 2 ml of dilute HCl were added to the test solution. An orange-red coloured precipitate indicates the presence of alkaloids.

**Wagner’s test:** 2 ml of Wagner’s reagent was added to 2 ml of test solution. The formation of reddish brown precipitate indicates the presence of alkaloids.

Tests for Glycosides:

**Conc. H$_2$SO$_4$ test:** To the extract add Conc. H$_2$SO$_4$ and allowed to stand for few minutes, it turned into reddish colour.

**Kellar Killiani test:** The extract was dissolved in glacial acetic acid and after cooling 2 drops of ferric chloride solution was added to it. These content were transferred to a test tube containing 2 ml of Conc. H$_2$SO$_4$. A reddish brown ring was observed at the junction of two layers.
Tests for Saponins:

**Foam test:** 0.01 g of crude extract was shaken vigorously in 2 ml of distilled water. Formation of honeycomb like froth persists for a few minutes indicate the presence of saponins.

2.3.5 Quantitative Estimation of Secondary Metabolites:

Different plant parts *viz.*, leaf, seed coat and cotyledon of *C. cajan* were analyzed quantitatively to find out the concentration of different secondary metabolites by following methods.

2.3.5.1 Alkaloids:

The alkaloid was estimated by the method of Harborne (1973). The acetic acid (5%) extract of the plant material was warmed up to 70° C and the pH-10 was made by NH₄OH and centrifuged at 5000 rpm. The precipitate was dissolved in ethanol (95%) and H₂SO₄. The alkaloid solution was mixed with 5 ml of 60% H₂SO₄ after 5 min, 5 ml of solution of formaldehyde in H₂SO₄ was added. The solution was read at 565 nm absorbance after 15 min the amount of alkaloids was calculated using the standard curve of Brucine.

2.3.5.2 Flavonoids:

The total Flavonoid was estimated following the method of Jia *et al.* (1999). The plant sample 0.5 - 1 g were homogenised in 10 ml of methanol and centrifuged. 1 ml of supernatant was taken in test tube and add the 0.25 ml of distilled water followed by addition of 75 µl of a 5% NaNO₂ solution. After 6 min 150 µl of a 10% AlCl₃ solution was added and allowed to stand for another 5 min before 0.5 ml of 1 ml NaOH was added.
The mixture was brought to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately against the blank at 510 nm using a spectrophotometer.

2.3.5.3 Glycosides:

The total glycoside was estimated according to the method of Huguchi and Hanssen (1997). The plant material was acid hydrolysed by 2.5 N HCl in the ratio of 1:10 (w/v) for 3 h on water bath. Further, 3 ml of aliquot containing 0.2 mg/ml of hydrolysed plant material was taken with 15 ml of Picrate reagent and diluted to 25 ml by ethyl alcohol. The reaction mixture is kept for 30 min and the absorbance was read at 490 nm against the reagent blank. The amount of glycosides was calculated using standard graph prepared from Digoxin.

2.3.5.4 Phenols:

The total phenols were estimated by Folin-Ciocalteau method (Malick and Singh, 1980). Plant samples of 0.5 - 1.0 g were homogenized with 5 ml of 80% of ethanol in pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min. The residue was extracted with 5 ml of 80% ethanol and centrifuged. The supernatants were evaporated to dryness. This dried plant extract was used to estimate the total phenols. Further, the residue was dissolved in 3 ml of distilled water containing 0.1 ml of plant extract and 0.5 ml of Folin-Coicalteau reagent was added. After 3 min of incubation, 2 ml of 20% Na₂CO₃ was added and placed the test tube in boiling water bath for 1 min then cooled and measured the absorbance at 650 nm against
the reagent blank. The content of total phenols was calculated using the
calibration curve of Gallic acid.

2.3.5.5 Steroids:

The total steroids were estimated according to the method of
Sanchez et al. (1972) modified by Rishi et al. (1976).

500 mg of plant material was hydrolysed by refluxing with 25 ml of
3N HCl at 60% for 4 h. Filter, the solid matter retained on the
Whatmann filter paper No. 1 was washed with half diluted aqueous NH₄OH
(pH 6.8 - 7.0). Then the residue was dried for 6 h and extracted for the
estimation of sterol in the Soxhlet extraction using chloroform. From this,
1 ml extract was taken and evaporated to dryness. Thus, the residue obtained
was dissolved in 4 ml H₂SO₄ and methanol reagent. The reaction was allowed
to proceed for 2 min (it is an optimum time require for the chromatophore to
develop a stable optical density). The absorbance was read at 450 nm in UV
spectrophotometer against a blank. The amount of steroids present in the
plant material was calculated using standard graph prepared from Diogenin.

2.3.5.6 Tannins:

The total tannins were estimated by Folin Denis method (Schanderi,
1970). 0.5 g of the powdered material was transferred to a 250 ml conical
flask. 75 ml of distilled water was added. The flask was heated gently and
boiled for 30 min, centrifuged at 2,000 rpm for 20 min and collected the
supernatant in 100 ml volumetric flask and made up the volume. 1 ml of the
sample extract was transferred to a 100 ml volumetric flask containing 75 ml
of water. Added 5 ml of Folin-Denis reagent, 10 ml of sodium carbonate solution and diluted to 100 ml with distilled water and shaken well. The blue colour intensity was measured in a spectrophotometer. Read the absorbance at 700 nm after 30 min and made a 30 times dilution of the sample with distilled water and prepared a standard graph by using 0 - 100 µg tannic acid. Calculated the tannic acid equivalents from the standard graph expressed as mg/100 mg.

2.3.5.7 Lignins:

The total lignins were estimated according to the method of Stafford (1960). Moisten 100 mg of oven-dried material was grind with ether until it is free from chlorophyll pigment. Centrifuge at 2000 rpm for 5 min and decant the supernatant. Wash the sediment with water, centrifuge and discard the supernatant. Add 2 ml of NaOH to the residue and extract at 70 - 80°C for 12 - 16 h. Cool and add the 0.45 ml of HCl and adjust the pH to 7 with NaOH. Make up the volume to 3 ml with water. Centrifuge at 2000 rpm for 5 min collect the supernatant. To 0.8 ml of extract add 0.8 ml of 0.1 M phosphate buffer pH 7.0. To 0.8 ml of extract add 0.8 ml of 0.1N NaOH (pH 12.3). Measure the absorbance at 350 nm.

Statistical Analysis:

The data of all measurements are means from three replications. Data and statistical significance of difference were evaluated with analysis of variance (ANOVA) using SPSS 10.0 package.
2.4 RESULTS AND DISCUSSION:

The following results on quantities of primary metabolites, preliminary screening and quantities of secondary metabolites in different parts of *C. cajan*. The results thus obtained are discussed in the light of the available literature.

2.4.1 Primary Metabolite contents in *C. cajan*:

The total contents of primary metabolites such as proteins, carbohydrates, lipids, DNA and RNA in leaf, seed coat and cotyledon of *C. cajan* were estimated in different methods and their results are shown in Fig. 2.1.

The total protein contents in the dried and powdered plant material of *C. cajan* were 22.2±0.21 mg/100 mg (leaf), 4.9±0.64 mg/100 mg (seed coat) and 68.0±0.52 mg/100 mg (cotyledon). The more amounts of proteins were found in cotyledon (Fig. 2.1). Similarly, Gepts *et al.* (2005) have reported the more contents of proteins in legumes. These proteins help to regulate blood sugar, body fluid, kidney, adrenaline, liver function and other aspects of metabolism.

The total carbohydrate contents in the dried and powdered plant material of *C. cajan* were 40.0±0.23 mg/100 mg (leaf), 0.94±0.62 mg/100 mg (seed coat) and 56.63±0.13 mg/100 mg (cotyledon). The maximum amounts of carbohydrates were found in cotyledon while the least in the seed coat (Fig. 2.1).
Fig. 2.1: Primary Metabolites in different parts of *C. cajan*
The quantitatively estimated the total lipid contents of *C. cajan* was 25.0±0.28 mg/100 mg (leaf), 42±0.72 mg/100 mg (seed coat) and 45.0±0.81 mg/100 mg (cotyledon). The maximum amounts of lipids were found in cotyledon while the least in the seed coat (Fig. 2.1).

The DNA amount varied from 0.51±0.12 to 5.45±0.16 mg/100 mg of plant material. The maximum amount of DNA was observed in cotyledon (5.45±0.16 mg/100 mg) and moderate amount was observed in leaf (1.75±0.45 mg/100 mg). The least amount of DNA was observed in seed coat (0.51±0.12 mg/100 mg) (Fig. 2.1).

The RNA amount varied from 0.80±0.56 to 8.25±0.81 mg/100 mg. The maximum amount of RNA was observed in cotyledon (8.25±0.81 mg/100 mg) and moderate amount was observed in leaf (0.96±0.12 mg/100 mg). The least amount of RNA was observed in seed coat (0.80±0.56 mg/100 mg).

### 2.4.2 Preliminary Screening for Secondary Metabolites in *C. cajan*

The Soxhlet successive crude extracts *viz.*, pet-ether, chloroform, ethanol and aqueous extracts of leaf, seed coat and cotyledon were qualitatively screened for the occurrence of various secondary metabolites such as alkaloids, flavonoids, glycosides, tannins, lignins, phenols, saponins and steroids by treating with various chemical reagents. The reactions with these reagents have shown the presence or absence of metabolites and are recorded in the Table – 2.1.
Table – 2.1: Distribution of Secondary Metabolites in different parts of *C. cajan*

| Secondary metabolites | Name of the test | Leaf | | | Seed coat | | | Cotyledon | | |
|-----------------------|------------------|------|------|------|--------|------|--------|--------|------|------|------|
|                       |                  | PE   | CHCl₃| Et-OH | Aq     | PE   | CHCl₃| Et-OH | Aq     | PE   | CHCl₃| Et-OH | Aq     |
| Phenols               | Ellagic acid test| +    | +    | +    | +     | +    | +    | +     | +     | +    | +    | +     | -      |
|                       | Phenol test      | +    | +    | +    | +     | +    | +    | +     | +     | +    | +    |         |         |
| Tannins               | Gelatin test     | -    | -    | +    | +     | -    | -    | +     | -     | -    | -    | +     | -      |
| Flavonoids            | Pews test        | +    | +    | +    | +     | +    | +    | +     | +     | +    | +    | +     | +      |
|                       | Shinoda test     | +    | +    | +    | +     | +    | +    | +     | +     | +    | +    | +     | +      |
|                       | NaOH test        | +    | +    | +    | +     | +    | +    | +     | +     | +    | +    | +     | +      |
| Lignins               | Labat test       | +    | +    | +    | +     | +    | +    | +     | +     | +    | +    | +     | +      |
|                       | Lignin test      | +    | +    | +    | +     | +    | +    | +     | +     | +    | +    | +     | +      |
| Steroids              | Libermann-burchard test | -  | -    | +    | +     | -    | -    | +     | +     | -    | -    | +     | +      |
|                       | Salkowski test   | -    | -    | +    | +     | -    | -    | +     | +     | -    | -    | +     | +      |
| Alkaloids             | Iodine test      | -    | -    | +    | +     | -    | -    | +     | +     | -    | -    | +     | +      |
|                       | Dragendroff’s test | -  | -    | +    | +     | -    | -    | +     | +     | -    | -    | +     | +      |
|                       | Wagner’s test    | -    | -    | +    | +     | -    | -    | +     | +     | -    | -    | +     | +      |
| Glycosides            | Kellar-kilani test | -  | +    | +    | +     | -    | +    | +     | +     | -    | +    | +     | +      |
|                       | Conc. H₂SO₄ test | -    | +    | +    | +     | -    | +    | +     | +     | -    | +    | +     | +      |
| Saponins              | Foam test        | -    | -    | -    | -     | -    | -    | -     | -     | -    | -    | -     | -      |

2.4.2.1 Phenols:

The preliminary test such as phenol and Ellagic acid tests showed positive to all extracts of leaf, seed coat and cotyledon. Whereas, aqueous extract of cotyledon shows negative to these test. Formation of muddy yellow colour by the Ellagic acid test shows the presence of trihydroxy phenolics like ellagic acid and gallic acid which are known to be the nuclear compounds of tannins (Bate and Smith, 1958). Similarly, the test solutions have also shown positive response to phenol test. It is known that ellagic acid is a potent inhibitor of mutagenicity and cytogenecity (Backer et al., 1985).

2.4.2.2 Tannins:

The ethanol and aqueous extracts of leaf shows presence of tannins by showing positive to the tests of tannins namely FeCl$_3$ and tannin tests. Similarly, ethanol extract of seed coat and cotyledon shows positive to above tests, remaining extracts shows negative to these tests. The earlier report of Duke (1981) supports the present results.

2.4.2.3 Flavonoids:

The pet ether, chloroform, ethanol and aqueous extracts of leaf, seed coat and cotyledon showed positive to the tests of flavonoids i.e., Shinoda, Pew’s and NaOH tests. The Shinoda and Pew’s tests showed developing deep cherry red colour indicating the occurrence of dihydrokaempferol flavonoids and deep red or magenta colour indicating the presence of flavones respectively. The positive response to different flavonoid tests
indicates the occurrence of more than one type of flavonoids. The earlier report of Duke (1981) supports the present results.

2.4.2.4 Lignins:

The test solution have shown positive response to lignin test by the development of a red colour due to the occurrence of dimeric cinnamic acid derivatives in the test solutions. The preliminary tests such as labbat and furfuraldehyde tests showed positive to all parts which indicated the presence of lignins. The extracts of C. cajan showed positive reaction to Gelatin test by formation of white precipitate. The earlier report of Duke (1981) supports the present results.

2.4.2.5 Steroids:

The ethanol and aqueous extracts of all parts of C. cajan shown positive response to steroids by the development of wine red colour precipitation and bluish green colour in the tests of Salkowski and Libermann and Burchard tests indicates the presence of steroids has reported by Duke (1981).

2.4.2.6 Alkaloids:

The ethanol and aqueous extracts were positive to the preliminary alkaloid tests i.e., iodine, Dragendroffs and Wagner’s reagents indicating the presence of alkaloids. The positive responses to iodine, Wagners and Dragendroffs to all tests confirms the occurrence of more than two types of alkaloids in the test solutions by the formation of yellow, white and orange
precipitation respectively. Duke (1981) has also shown the occurrence of alkaloids in *C. cajan*.

**2.4.2.7 Glycosides:**

The chloroform, ethanol and aqueous extracts of all parts of *C. cajan* indicated the presence of glycosides by giving characteristic reaction with the tests of glycosides namely Conc. H$_2$SO$_4$ and Kellar Killiani test by formation of reddish brown colour ring at the junction of two liquids and formation of the reddish colour respectively, indicating more than one type of glycosides may be present. Presences of glycosides are seen in *C. cajan* by Duke (1981).

**2.4.2.8 Saponins:**

The leaf, seed coat and cotyledon extracts were completely devoid of saponins. The foam and haemolysis tests showed the absence of saponin. According to Segelman and Fransworth (1968) if tannin are present in the plant extracts the haemolysis test gives negative result.

The preliminary screening tests have revealed that the presence of the various groups of secondary metabolites such as phenols, tannin, flavonoids, alkaloids, glycosides, lignins and steroids in the leaf, seed coat and cotyledon.

**2.4.2.2 Secondary Metabolite contents in *C. cajan*:**

The total contents of secondary metabolites such as total phenols, flavonoids, tannins, lignins, steroids, glycosides and alkaloids in leaf, seed coat and cotyledons of *C. cajan* are estimated from different methods and their results are as shown in Fig. 2.2.
Fig. 2.2: Secondary Metabolites in different parts of *C. cajan*
It is observed that the total phenols were the most abundant group of secondary metabolites estimated from the various parts of *C. cajan* (Fig. 2.2). The content of phenols in leaf, seed coat, cotyledon were 232.45±0.82 mg/100 mg, 308.15±0.85 mg/100 mg and 3.7±0.18 mg/100 mg respectively. The highest amounts of phenols were observed in the seed coat. Bhattacharya and Chenchaiah (2007) have observed the more contents of phenolic compounds in seed coat of *C. cajan*. Similar result was reported by Agenieszka and Ewa Cisk (2002) in seed coats of *Pisum sativum*. Some studies have reported that phenolic compounds which inhibit the activities of α-amylase, α-glucosidase and protease, provide an attractive target for the development of potential therapeutic agents to treat diabetes, pancreatitis, coagulation and neoplastic diseases and also as a source antioxidants (Chiasson *et al.*, 2002; Frecre *et al.*, 2000; Wang, 2001).

The total flavonoids were found to vary from 0.18±0.10 to 350.43±0.65 mg/100 mg of plant material. The maximum amounts of flavonoids were found in leaf (350.43±0.65 mg/100 mg). Moderate amounts were in seed coat (290.13±0.62 mg/100 mg) and comparatively very less amount in cotyledon (0.18±0.10 mg/100 mg) (Fig. 2.2). The results revealed that leaf has a more amount of flavonoids. These findings are supported by Yuan-Gang Zu *et al.* (2006) have observed more contents of flavonoids and determined the four flavonoids in leaves of pigeon pea. Okwu (2004) reported the flavonoids are a source of antioxidants and have strong anti-cancer activity.
The total tannins content varied from 1.92±0.15 to 251.29±0.10 mg/100 mg of plant material. The maximum amounts of tannins were found in seed coat 251.29±0.10 mg/100 mg. Moderate amounts were in leaf 190.0±0.98 mg/100 mg and comparatively very less amount in cotyledon 1.92±0.15 mg/100 mg (Fig. 2.2). Similarly, Ayodele and Kigbu (2005) have reported the total tannin contents in seed of Cajanus cajan had 5.50±0.05 mg/g while Sterculia setigera and Vigna dekindtiana had 4.24±0.04 mg/g and 16.16±0.05 mg/g respectively. In the present study, highest value of tannins was recorded in seed coat. Similarly, Agenieszka and Ewa Cisk (2002) have reported more contents of tannin in the seed coat of pea (Pisum sativum). Okwu (2004) have reported tannins serves as astringent properties for healing of wound, inflaming mucous membrane and also source of antioxidant.

The total alkaloids content varied from 0.09±0.01 to 118.32±0.85 mg/100 mg of plant material. The maximum amounts of alkaloids were found in leaf 118.32±0.85 mg/100 mg whereas, the least amounts were observed in seed coat and cotyledon 0.82±0.12 and 0.09±0.01 mg/100 mg respectively (Fig. 2.2). Mbagwu et al. (2011) have reported the contents of alkaloid in leaves of four edible legumes (Vigna subterranean, Glycine max, Arachis hypogeal and Vigna unguculata).

The total glycosides content varied from 0.19±0.01 to 252.43±0.95 mg/100 mg of plant material. The maximum amounts of glycosides were found in leaf 252.43±0.95 mg/100 mg whereas, the least amounts were found in seed coat and cotyledon 0.190±0.01 and 115.34±0.81 mg/100 mg respectively (Fig. 2.2). Some
studies have reported the glycosides are useful for cardiac diseases (Harborne, 1973; Brian Leibovitz and Jennifer Ann Mueller, 1993).

The total steroids content varied from 0.21±0.08 to 211.37±0.92 mg/100 mg of plant material. The maximum amounts of steroids were found in leaf 211.37±0.92 mg/100 mg whereas the least amounts were found in seed coat and cotyledon 0.63±0.05 and 0.21±0.08 mg/100 mg respectively (Fig. 2.2). Similarly, Muhammad et al. (2009) have reported the 3.30 mg/g steroids in seed oil of chickpea. Plant sterols possess a broad spectrum of therapeutic effects in animals and humans. In humans, consumption of plant-derived sterols, particularly β-sitosterol, reduces blood pressure serum cholesterol levels and the risk of chronic heart diseases (Ling and Jones, 1995; Clark, 1996; Moreau et al., 2002). In addition, phytosterols are known as antipolymerization factors and as antioxidants, especially those containing an ethylidene group in the aliphatic side chain (Δ7-Δ7-avenasterols), in vegetable oil at frying temperature (Wang et al., 2002).

The total lignins content varied from 0.15±0.06 to 200.0±0.82 mg/100 mg of plant material. The maximum amounts of lignins were found in seed coat (200.0±0.82 mg/100 mg). Moderate amounts of lignins were present in leaf, (198.3±0.73 mg/100 mg) and comparatively very less amount in cotyledon respectively (Fig. 2.2). Similarly, Morrison et al. (1995) have observed 236.0 gm Kg⁻¹ lignins in brown cow pea seed coat. Banerji (1998) have reported the lignins in Vitex negundo plant. It is phenolic polymers are characterised as active principles for its anti stress properties. Vinardell et al. (2008) have reported the lignins acts as antioxidants.
Of these secondary metabolites quantitatively estimated from the various parts of *C. cajan* there is a considerable variation in their content of occurrence among leaf, seed coat and cotyledon samples. It is observed that leaf possesses higher amounts of flavonoids, glycosides, alkaloids and steroids compared to other parts of the plant. Similarly, seed coat is a rich source of secondary metabolites like phenols, tannins, lignins, flavonoids and these are found very negligible amount in cotyledon (Deshpande *et al.*, 1982; Agnieszka and Ewa, 2002). The outcomes of this phytochemical screening thus suggest that seed coats are rich source of secondary metabolites are highly recommended in everyday diet of man and health benefits.