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SCREENING OF TRACE METALS AND SECONDARY METABOLITES FROM *Cajanus Cajan* (L.) Mill sp. AND STUDY THEIR ANTIMICROBIAL EFFICACY

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ABSTRACT

The present study focused on the Phytochemical constituents, trace metals concentrations, and antimicrobial activity of ethanolic extract of Cajanus cajan leaves were investigated. The plant leaves which were collected from the Tiruchirappalli district, southern India. The identification of secondary metabolites revealed the presence of the bioactive compounds, such as steroids, sugars, alkaloids, phenol, flavonoids, tannins, glycoside and amino acids. Likewise, the primary metabolites such as chlorophylls contents, carbohydrate, proteins and lipids were also presented in the ethanolic extract of C. cajan leaves. The antimicrobial sensitivity against C. cajan using the disc diffusion method. The ethanol extracts of the plant leaves exhibited the higher zone of inhibition against bacterial strains than fungal strains. The trace metal concentrations were analyzed form the powered plant leaves by 797 VA Computrace voltametry, Metrohm. The average concentrations of Cd, Cr, Cu, Fe, Ni, Pb and Zn were below detectablelimit (BDL), BDL, 0.06, 0.42, BDL, BDL and 0.33 mg kg-1, respectively. The bioactive secondary metabolites were responsible for these antimicrobial activities.

KEYWORDS: Cajanus cajan, Antimicrobial efficacy, Photochemistry, Trace metals.

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 result from the combination of secondary metabolic products that are present in the plant (Ahmed John and Koperuncholan 2012). 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 a nalogs 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 (Ahmed John and Koperuncholan 2012a and Fazal Mohamed et al. 2011). 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, with the objective of observing and analyzing their respective chemical constituents trace metal and antimicrobial efficiency.

MATERIALS AND METHODS

Plant Material

Fresh leaves of Cajanus cajan was collected from Tiruchirappalli district of Tamil Nadu during the period of October – November 2015. The shade dried plant powder (100 g) was loaded in the thimble of Soxhlet apparatus. It was fitted with appropriate size round bottom flask with 250 ml absolute ethanol, and upper part was fitted with condenser. Constant heat was provided by Mantox heater for recycling of the solvent. After complete extraction, the extract in round bottom flask was transferred into clean and pre-weighed universal tubes. Universal tubes containing extracts were weighted and noted down and finally, the percentage yield was calculated. Percentage yield was calculated as dividing initial weight of raw material taken by final weight of extract. Then, the extracted solution was dried by hot air oven at 50 °C for 48 h.

Quantitative estimation of primary metabolites

The dried and powdered plant material of C. cajan leaf, was used for quantitative estimation of primary metabolites such as proteins, carbohydrates and lipids by using different methods are as follows.

Chlorophylls

The chlorophyll pigments in the leaves were estimated following the method of Arnon. After pre-cleaning, weighted fresh leaf material was homogenized and extracted thrice in chilled 80% acetone (v/v). The volume of the acetone extract was made up to a known one and the optical density was read at 645 nm and 663 nm wavelengths on a spectrophotometer. The concentration of the chlorophyll pigments was calculated and is expressed in mg/g fresh weight.

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 40 C 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 upto 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.

Carbohydrates

Total carbohydrate was estimated by Anthrone reagent (Yemm and Willis, 1954). Plant liquot (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.

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 H2SO4 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.

Identification tests for secondary metabolites

The phytochemical constituents were analyzed from the Cajanus cajan plants by the standard procedures (Koperuncholan and Ahmed John 2011).

Steroids

A 3 ml of test solution and minimum quantity of chloroform was added with 3-4 drops of acetic anhydride and one drop of concentrated H_2SO_4. Purple color thus formed changes into blue or green color indicating the presence of steroids.

Tritrepenoids

A 3 ml of test solution was added with a piece of tin and 2 drops of thionyl chloride. Formation of violet or purple colour indicates the presence of Tritrepenoids.

Reducing Sugars

A 3 ml of test solution was added with a 2 ml of Fehling's reagent and 2 ml of water. Formation of reddish orange color indicates the presence of reducing sugar.

Sugars

A 3 ml of the test solution was added with very small quantity of anthrone reagent and a few drops of concentrated H_2SO_4 and heated. Formation of green or purple color indicates the presence of sugars.

Alkaloids

A 3 ml of test solution was taken with 2N HCl. Aqueous layer formed was decanted and then added with one or a few drops of Mayer's reagent. Formation of white precipitate or turbidity indicates the presence of alkaloids.

Phenols

A 3 ml of test solution in alcohol was added with one drop of neutral ferric chloride (5%) solution. Formation of intense blue color indicates the presence of phenols.

Flavonoids

A 3 ml of test solution in alcohol was added with a bit of magnesium and one (or) two drops of concentrated HCl and heated. Formation of red or orange color indicates the presence of flavonoids.

Saponins

A 3 ml of test solution was added with water and shacked. Formation of foamy lather indicates the presence of Saponins.

Tannins

A 3 ml of test solution was added with water and lead acetate. Formation of white precipitate indicates the presence of tannins.

Anthroquinones

A 3 ml of test solution was added with magnesium acetate. Formation of pink color indicates the presence of anthroquinones.

Glycoside

Glycosides are compounds which upon hydrolysis give rise to one or more sugars (glycones) and a compound which is not a sugar (aglycone or genine). To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added, and observed for a reddish brown coloration at the junction of two layers and the luish green color in the upper layer.

Amino Acids

A 3 ml of test solution was added with 1% ninhydrin in alcohol. Formation of blue or violet color indicates the presence of amino acids. Catechins: A 3 ml of test solution in alcohol was added with Ehrlich reagent and a few drops of concentrated HCl. Formation of pink color indicate the presence of catechins.

Analysis of trace metals in plants

The Cajanus cajan plant sample was collected from the Tiruchirappalli district, Tamil Nadu. The plant leaves were carefully removed and washed with sterile distilled water, separately. The cleaned leaves were dried in shadow area and were grinned with agate mortar and pestle. The powered plant samples were stored in sterile plastic container. The 1 g of powdered plant samples was treated with aqua-regia mixture (hydrochloric acid + nitric acid) in Teflon bomb

and was incubated at 140 °C for 2-3 days (Koperuncholan and Ahmed John 2011a). After incubation, the reaction mixture was filtered with nitrocellulose (0.45 µm) filter paper by Millipore vacuum filtration unit. Then the extraction was test for trace metals (Fe, Cu, Zn, Pd, Cd, Cr and Ni) analysis. The extraction of the studied metals in the solutions was determined by the 797 VA Computrace voltammetry, Metrohm.

Testing of antimicrobial activity

The test strains were: Aeromonas liquefaciens MTCC 2645 (B1), Enterococcus faecalis MTCC 439 (B2), Micrococcus luteus NCIM 2871 (B3), Salmonella typhimurium NCIM 2501 (B4), Candida albicans MTCC 1637 (F1) and Cryptococcus sp. MTCC 7076 (F2). The cultures were obtained from MTCC, Chandigarh and NCIM, Pune, India (Vignesh et al., 2015a). Microbial strains were tested for antimicrobial sensitivity using the disk diffusion method (Anitha et al., 2011; Vignesh et al., 2012a; Vignesh et al., 2012b). The antibacterial and antifungal activities of test samples were analyzed against certain microorganisms on muller hinton agar (MHA) and potato dextrose agar (PDA), respectively (Vignesh et al., 2013; Vignesh et al., 2014). The solvent extracted samples were dissolved in concentrated DMSO. A sterile cotton swab was used to inoculate the bacterial suspension on surface of agar plate. The three different concentrations (2.5, 5 & 10 mg/ml) of sample were poured into disk and placed on agar plates, separately. For negative control study, the DMSO was used (Lakshmi praba et al., 2013). The plates were incubated at 37±1°C for 24–48 h (for bacteria) and 25 ±1°C for 48-72 h (for fungi) (Muthukumar et al., 2015). After incubation, the zone of inhibition was measured with ruler. The assays were performed in triplicate and the average values are presented (Vignesh et al., 2015b). Methicillin – 10mcg (for bacteria) and Itraconazole – 10mcg (for fungus) was used as positive control (Beevi et al., 2012; Pandiyarajan et al., 2013). All the media, standard discs and sterile disc were purchased from Hi-Media (Mumbai, India).

RESULTS AND DISCUSSION

The following results on quantities of primary metabolites, preliminary screening of secondary metabolites, trace metal concentration and antimicrobial efficiency in Cajanus cajan. The results thus obtained are discussed in the light of the available literature. The ethanolic leaf extract of C. cajan screened for the presence of various biologically active primary and secondary metabolites. The quantitative results revealed of primary metabolites of chlorophylls A, chlorophylls B, Total chlorophylls, carbohydrate, proteins and lipids

presented in 0.419, 0.852, 1.271, 22.2, 40, 25 mg/g respectively (Table.1). Likewise, the presence of secondary metabolites are steroids, sugars, alkaloids, phenol, flavonoids, tannins, glycoside and amino acids (Table.2). A common role of secondary metabolites in plants is defense mechanisms. They are used to fight off herbivores, pests and pathogens. Although researchers know that this trait is common in many plants it is still difficult to determine the precise role each secondary metabolite.

Secondary metabolites are used in anti-feeding activity, toxicity or acting as precursors to physical defense systems. 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 (Koperuncholan M and Manogaran 2015). 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 (Koperuncholan M, 2015). 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 (Koperuncholan et al. 2010) supports the present results. 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. 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). 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.

Sinthiya and Koperuncholan (2015) has also shown the occurrence of alkaloids in C. cajan. 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_2SO_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). The leaf, seed coat and cotyledon extracts were completely devoid of saponins. The foam and haemolysis tests showed the absence of saponin. According to tannin is 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.

Some of the trace metals are essential for plant growth whereas many of them affect the plant physiology. Especially, the role of trace metal pollutants causing injury to plants either by direct toxic effect or modifying the host physiology rendering it more susceptible to infection (Ramesh et al., 2014) which leads to affects the photosynthesis process, growth and their efficiency. The mean concentrations of metals such as Cd, Cr, Cu, Fe, Ni, Pb and Zn were below detectable limit (BDL), BDL, 0.06, 0.42, BDL, BDL and 0.33 mg kg-1, respectively (Table 3). Through the natural process of bio-magnifications, minute quantities of metals become part of the various food chains and concentrations become elevated to levels which can prove to be toxic to human, animal, plant and other living organisms (Koperuncholan and Ahmed John, 2011). In particularly, higher trace metal in the plants caused progressive reduction in the photosynthetic ability of leaves, closure of leaf stomata, and productivity of plants, ascorbic acid content and chlorophyll content (Ramesh et al., 2014).

The antimicrobial activity of C. cajan was examined with various microorganisms using the disk diffusion test. The results of the antimicrobial activities are summarized in Table 4. The three tested concentrations such as 2.5, 5 & 10 mg/ml produce zone of inhibition on MHA and PDA plates for bacteria and fungi, respectively. In the present study, higher (10 mg/ml) concentration of sample got greater sensitivity than (2.5 & 5 mg/ml) lower concentration in most of the microorganisms. In bacteria, the ethanol extract samples were most effective against Salmonella typhimurium (B5) while the smaller effect was noticed from Micrococcus luteus (B4). In fungi, the test sample was effective against Trichophyton rubrum (F4). There

is no antimicrobial activity in solution devoid of sample used as a vehicle control (concentrated DMSO), reflecting that antimicrobial activity was directly related to the sample. Suresh et al. (2008) reported the best antimicrobial activity of Rauvolfia tetraphylla, which showed maximum activity against E. coli and Enterobacter aerogenes, and various tested fungi such as A. niger and Penicillium sp, were found to be more sensitive to crude extract when compared to others. Several phytoconstituents such as terpenoids (Koperuncholan and Ahmed John, 2011), flavonoids (Ahmed John and Koperuncholan, 2012a) and tannins (Koperuncholan, 2015) are effective against certain microorganisms. The results of the present investigation clearly demonstrate the antibacterial and antifungal activities of the ethanol extracts of the leaves.

Table: 1 Qualitative phytochemical constituent of Cajanus cajan

Phytochemical Constituents	**Ethanol**
Steroids	+
Triterpenes	-
Reducing sugars	-
Sugars	+
Alkaloids	+
Phenolics	+
Catechins	-
Flavonoids	+
Saponins	-
Tannins	+
Anthraquinones	-
Glycoside	+
Amino acids	+

+ = Present; - =Absent

Table 2. Quantitative phytochemical constituent of Cajanus cajan

Biochemical constituents	**Cajanus cajan (mg/g)**
Chlorophyll A	0.419
Chlorophyll B	0.852
Total Chlorophyll	1.271
Protein	22.2
Carbohydrate	40.0
Lipid	25.0

Table 3. Concentration of trace metals in Cajanus cajan

Sampling Site	Sample Name	Cd	Cr	Cu	Fe	Ni	Pb	Zn
Tiruchirappalli Tamil Nadu	Cajanus cajan	0.07	BDL	0.26	0.72	BDL	BDL	0.43

BDL – Below detectable limit

Table 4. Antimicrobial activity of the different solvent extracts of Cajanus cajan leaves

S.No	Test Microorganisms		Ethanol extract mg/ml			PC	Diseases	Route of Transmission
Bacteria			2.5	5.0	10.0	10 mcg		
1.	Aeromonas liquefaciens	B1	9	10	12	14	Wound Infections / Gastroenteritis	Water / Food
2.	Enterococcus fecalis	B2	11	12	14	8	Endocarditis / Epididymal Infections	Water / Food
3.	Klebsiella pneumoniae	B3	12	13	16	28	Acute diarrhoea / Dysentery	Water / Food
4.	Micrococcus luteus	B4	10	11	13	38	Skin & Pulmonary infections	Soil / Water / Air / Food
5.	Salmonella typhimurium	B5	11	12	14	0	Typhoid	Water / Food
6.	Vibrio cholarae	B6	10	13	16	16	Cholera	Water / Food
Fungi								
7.	Candida albicans	F1	11	13	16	10	Skin infection / Gastrointestinal tract Infection	Air / Wound / Soil / Water
8.	Cryptococcus sp.	F2	12	14	17	9	Bronchiectasis / Endophthalmitis.	Air / Wound / Soil / Water
9.	Microsporum canis	F3	11	12	14	9	Tinea capitis /Ringworm	Air / Wound / Soil / Water
10.	Trichophyton rubrum	F4	10	12	15	7	Tinea corporis / Tinea pedis	Air / Wound / Soil / Water

PC - Positive Control (Using antibiotic disc; Bacteria – Methicillin (10mcg/disc); Fungi – Itraconazole (10mcg/disc)

Samples – 2.5, 5, 10 mg/ml (disk)

CONCLUSION

This study shown that all the primary and secondary metabolites were present in the Cajanus cajan. The C. cajan were absorbed the chemicals and metals which may affect the growth and biochemical properties of the plants. In antimicrobial study, all the microbial strains represent higher sensitivity to the higher concentration for the test sample when compared to the positive control except bacterial strains. Hence, we conformed that the C. cajan may affect the fungal strains in the extent and will act as an alternative antibiotic in the near future for fungal diseases.

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