Analysis of anti-nutritional factors and polysaccharide isolation from the seeds of *Leucaena leucocephala*

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Abstract: Anti-nutritional factors are natural compounds present in plants, usually plant secondary metabolites. Mimosine is the major anti-nutrient present in *L. leucocephala* seeds. Plant carbohydrates are stored as free sugar and polysaccharides. Polysaccharide has wide application in various fields and its extraction plays a major role for optimum utilization. In this study, several anti-nutritional factors were identified and polysaccharide extraction has been optimized using alkaline and alkaline-alcohol combination solvents.

Index term: Anti-nutritional factors, polysaccharide, *Leucaena leucocephala,* seed.

1. INTRODUCTION: *Leucaena leucocephala is* popularly known as subabul, river tamarind, white lead tree, miracle tree. The specific name 'leucocephala' comes from 'leu' meaning white and 'cephala', meaning head, referring to the flowers. The origin of this plant is Southern Mexico and Northern Central America but presently we can find this plant throughout the tropical regions. *L. leucocephala*, a member of the Mimosoideae family, is a leguminous tree/shrub that can grow rapidly in arid climates **Shelton and Brewbaker, 1994.** According to **M.Tuda *et al*., 2009** this is 'conflict species'. This plant was listed as one of the 100 worst invasive alien species in the world. The seed of *L. leucocephala* contains many unsaturated fatty acids as analysed by GLC. The unsaturated fatty acids include linolic acid (64.5%), Palmitic acid (17.9%), Oleic acid (11%), stearic acid (6.1%) and lauric acid (1.7%). Recently seed oil is used in biomembrane modelling **Dr Shailendra Badal 2017.** *L. leucocephala* posses some allelopathic chemicals majorly mimosine and act as potential bio-herbicide. In weed control management seeds are more potential than leaves or other parts **M. Safwan Ishak *et al.*, 2016.** The family Leguminosae is one of the main plant families on earth, its endosperm containing galactomannan but the ratio of galactose and mannan varies from species to species. The researcher gained interest in *L. leucocephala* due to its ingenious property.

2. MATERIAL AND METHODS:

2.1 Materials: Dry seeds of *L. leucocephala* (river tamarind), acetic anhydride, Anthrone's reagent, ammonia, bovine serum albumin, chloroform, Cu reagents, ethanol, Fehling's solution, ferric chloride, FC reagent, hexane, hydrochloric acid, methanol, magnesium ribbon, nitric acid, potassium permanganate, phosphomolybdic acid reagent, potassium thiocyanate, conc. sulphuric acid, sodium hydroxide, trichloroacetic acid, sodium sulphate, Wagner's reagent.

2.2 Methods

a) Aqueous Extraction: 10% extract of dry whole seed powder was done with distilled water. The sample was extracted on a heating magnetic stirrer with constant stirring using a magnetic stirrer for 30 mins. The solution was centrifuged at 5,000 rpm for 10 mins. The supernatant was used for the assay.

Methanol Extraction: 5% extract of dry whole seed powder was done by maceration for 7days with intermittent agitation. The solvent used was methanol. The solution was centrifuged at 5,000 rpm for 10 mins at room temperature. Pellet was discarded and the supernatant used for the assay.

b) 5% extract of seed endosperm was done with 0.1N, 0.01N NaOH and different percentage of ethanol (20%, 40%, 60%, and 80%). The sample was extracted for 3 hours with constant stirring using a magnetic stirrer at room temperature. The supernatant obtained by centrifugation at 7000rpm for 20 mins, used for assay of sugar and protein. Pellet was dried and stored.

5% and 10% extract of seed endosperm was done with a combination of 0.01NaOH and different percentage of ethanol (20%, 40%, 60%, and 80%). The sample was extracted for 3 hours with constant stirring using a magnetic stirrer. The supernatant was obtained by centrifugation at 7000rpm for 20 mins, used for assay of sugar and protein. Pellet was dried and stored.

100 mg of the pellet obtained from (20% ethanol in 0.01N NaOH extraction) taken in 10 ml of different percentage of H_2SO_4 (100%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 1%). The sample was hydrolysed with constant stirring using a magnetic stirrer for complete solubilisation of pellet. Supernatant used for the estimation of hydrolysed sugar by Anthrone's method and values is converted into polysaccharide.

2.3 Analysis of anti-nutritional factors

Test for flavonoid: a) 2-3ml of methanol extract was taken with a piece of magnesium ribbon and 1 ml of conc.H_2SO_4.was added. Pink colouration indicates the presence of flavonoid **Kumar *et al.*, 2007**.

b) 2ml of methanol extract was reduced to dryness in the boiling water bath. The residue was treated with dil. NaOH followed by dil. HCl. Yellow solution with NaOH turn colourless with dil HCl confirms flavonoid **Onwukaeme *et al.*, 2007**.

Test for saponin: To 0.5 ml of aqueous extract, 5ml of dist. water was mixed and shaken vigorously. Persistence of frothing indicates the presence of saponin **J Parekh and S.V Chanda 2007**.

Test for phenol: Methanol extract was spotted on filter paper. To that add one drop of phosphomolybdic reagent and expose to ammonia vapour. The blue colouration of the spot confirms phenol **Kumar *et al.*, 2007**.

Test for tannin: 2 ml of methanol extract was taken to that 2ml of 10% ferric chloride was added. Green colouration of the solution indicates the presence of tannin **Sasidharan *et al.*, 2011**.

Test for phlobatannin: 2ml of aqueous seed extract was boiled with 2ml of 1% HCl. Deposition of a red precipitate was not seen indicates the absence of phlobatannin **Edeoga *et al.*, 2005**.

Test for reducing sugar: To 5ml of aqueous extract add 25ml of dil. H_2SO_4 and boiled for 15 min then cool and neutralize to pH 7 with 10% sodium hydroxide followed by addition of 5ml Fehling's solution. Presence of brick red precipitate indicates the presence of reducing sugar **Sasidharan *et al.*, 2011**.

Test for steroid: To 1ml of methanol extract add 1ml of chloroform. To that mixture add 2-3 ml of acetic anhydride followed by 2-3 drops of conc.H_2SO_4. The dark green colour was not seen indicates the absence of steroid **Kumar *et al.*, 2007**.

Test for terpenoid: To 1ml of methanol extract add 1ml of chloroform. 2ml of acetic anhydride was added followed by 2-3drops of conc.H_2SO_4. No pink colouration indicates the absence of terpenoid **Kumar *et al.*, 2007**.

Test for volatile oil: 2 ml of the aqueous extract with 0.1ml of dil. NaOH and a small quantity of dil. HCl. No white precipitate formed confirms the absence of volatile oil **Dahiru *et al.*, 2006**.

Test for Oxalate: One gram dry seed powder was extracted with 75 ml of 3 M H_2SO_4 in a conical flask and stirred using shaker incubator at 37°C for 60 mins. The solution was filtered; 25 ml of obtained filtrate was titrated against 0.1 N $KMnO_4$. The appearance of a faint pink colour indicates the presence of oxalate **Pramodini Rout and Uday Chand Basak 2014**.

Test for alkaloid: 5% extract of seed powder was carried out using 1% HCl. The sample was extracted for 30 mins with constant stirring in magnetic stirrer at room temperature. The solution was filtered. In a test tube, 2 ml of above filtrate was heated, to it, 5-6 drops of Wagner's reagent was added. Brick red precipitation obtained confirms alkaloid **Chanda *et al.*, 2006**.

Test for phytate: 3 gm of powdered sample was taken with 25ml of 10% TCA in 125ml conical flask and shaken for 2 hours. The mixture was centrifuged at 3000 rpm for 20 min. Pellet was discarded and 10ml of supernatant was mixed with 4ml of $FeCl_3$. The solution was heated in a boiling water bath for 45 min. To get the clear supernatant to add 2-3 drops of 3% sodium sulphate in 10 % TCA. Centrifugation was done at 3000 rpm for 15-20 min and the clear supernatant was decanted. The precipitate obtained was washed with twice with 25 ml of 10% TCA and centrifuged after cooling to room temperature. The precipitate was dispersed in 3 ml of 1.5N NaOH and made the volume up to 30 ml with dist. water. The solution was boiled for 30 min and filtered. The precipitate obtained was washed twice with hot water then dissolved in 40 ml of conc. HNO_3.5% dilution of the above solution was made followed by addition of 20 ml of 1.5M potassium thiocyanate. Presence of pink colour indicates the presence of phytate **Pramodini Rout and Uday Chand Basak 2014.**

2.4 Estimation of polysaccharide and protein.

a) Assay of sugar was done by Anthrone's Method according to **Hedge, J E and Hofreiter, B T 1962**. The reaction mixture composed of 1ml of extracted sample and 4ml of Anthrone's Reagent kept in boiling water bath for 10 mins. The mixture was cooled and absorbance was read at 620nm against a blank. Sugar was measured as glucose equivalents in g/100g of dry sample.

b) Protein assay was done according to **(Lowry *et al.*, 1951)**. 1ml of extract and 5ml of copper reagent was incubated for 10 min at room temperature followed by 0.6 ml of F.C reagent. The mixture is allowed to stand for 20 mins to develop the colour. Absorbance was measured at 660nm against a blank. Total protein content was expressed in g/100 g dry weight of a sample as Bovine serum albumin equivalent.

3. RESULT AND DISCUSSION:

Seeds of *L. leucocephala* has several anti-nutritional factors and listed in table1. Oxalate, alkaloid, saponin, phenol, flavonoid, tannin and phytate was present whereas, steroid, terpenoid, volatile oil, phlobatannin were absent in the seed of *L.leucocephala*. Presence of alkaloid and saponin gives antimicrobial property to plants **J Parekh and S.V Chanda 2007.** Anti-nutrient likes tannin influence the bioavailability and digestibility of nutrient and minerals **Bruna Carbas *et al.*, 2020.**

Table1 Analysis of anti-nutritional factors from seeds of *L.leucocephala*

Test for	Observation	Inference
Oxalate	The appearance of pale pink colour	Presence of oxalate
Alkaloid	The appearance of brown-red precipitation	Presence of alkaloid
Saponin	Persistence of frothing	Presence of saponin
Phenol	The appearance of blue colouration.	Presence of phenol
Flavanoid.	Red colouration was observed	Presence of flavanoid
Flavanoid.	Yellow colouration with dil. NaOH turns colourless with dil. HCl	Presence of flavanoid
Tannin.	Green colouration was observed	Presence of tannin.
Phytate	Pink colouration was observed	Presence of phytate.
Phlobatannin	No formation of red precipitation	Absence of phlobatannin
Reducing sugar.	Formation of brick red precipitation	Presence of reducing sugar
Steroid.	No dark green colouration was observed.	Absence of steroid
Terpenoid	No pink colouration was observed	Absence of terpenoid.
Volatile oil	No white precipitation is seen	Absence of volatile oil

Seed endosperm extract with different alkali solvent and different percentage of ethanol, a maximum protein obtained was 18.40g/100g from 0.01N NaOH. With combination of alkali and alcohol (0.01N NaOH + different percentage 20%, 40%, 60% and 80%) of ethanol, maximum protein 19.5357g/100g obtained from 0.01N NaOH + 20% ethanol Figure 1.

Seed endosperm extract with different alkali solvent and different percentage of ethanol, maximum soluble sugar 6.57g/100g from 0.01N NaOH. With combination of alkali and alcohol (0.01N NaOH + different percentage 20%, 40%, 60% and 80%) of ethanol, maximum soluble sugar 8.2457g/100g obtained from 0.01N NaOH + 40% ethanol Figure.2.When the percentage of extract increased from 5% to 10% yield does not increase both for protein and soluble sugar.

The residue obtained from 0.01N NaOH + 20 % ethanol extract is maximum and solubilised with different percentage of concentration H_2SO_4 (100%60%, 50%, 40%, 30%, 20%, 10%, 5%, 1%). Yield of insoluble polysaccharide (8.55%) with 50% concentration of H_2SO_4 Table 3

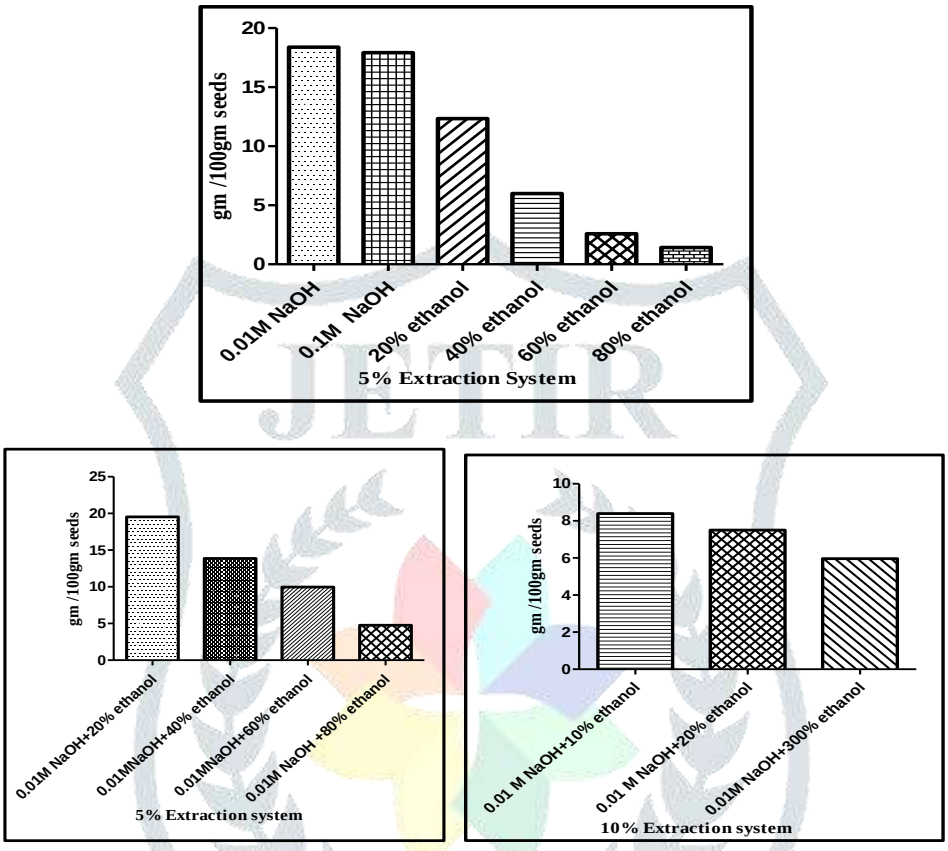

Figure1 Amount of soluble protein obtained from seed endosperm of *L.leucocephala*

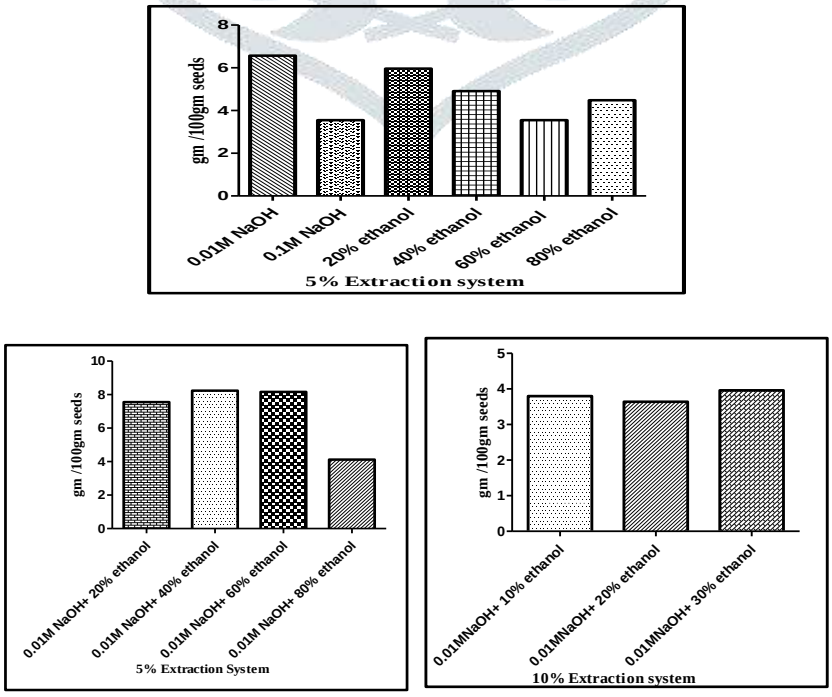

Figure 2 Amount of soluble sugar obtained from seed endosperm of *L.leucocephala*

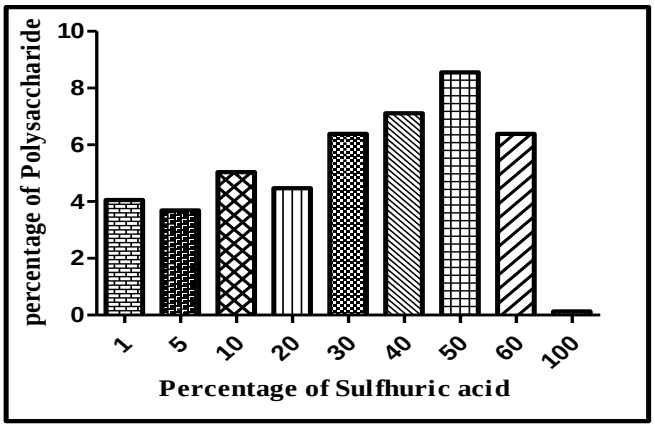

Figure 3 Amount of polysaccharide in the residue of alkali extract of defatted seed endosperm of *L. leucoceplaha*

Extraction technique plays a vital role to get optimum phytochemicals. Drying and grinding of plant materials like milling, crushing using mortar and pestle plays an important role. Choice of solvents depends upon several factors like solubility, extraction efficiency, reactions with phytochemicals etc. Usually, water is a universal solvent to extract plant products with antimicrobial activity, acetone dissolves many hydrophilic and lipophilic components. Ethanol is used to extract polyphenols compare to aqueous extract may be due to their polarity and ability for cell wall and seed degradation. Often alcohol-alkali mixture is used to get better extraction efficiency. Chloroform, hexane, methanol were used to extract terpenoids and tannins. For the extraction of fatty acid and coumarins usually, ether is used **S. Velavan 2015** Physical properties of which include solubility, the viscosity may affect bioavailability and extractability. Extraction with an acid like hydrochloric acid or trichloroacetic acid may cause hydrolysis of polysaccharide hence yield will be lower **Zhi- Peng Zhang *et al.*, 2017.**

20 % polysaccharides present in seeds of *L.leucocephala.* Presence of galactomannan is reported to be present in seed endosperm composed of a linear chain of β(1-4) D mannose substituted withα-D galactose at O-6(1.3:1). The water-soluble polysaccharide was found a maximum degree of substitution with hydrophilic and swelling properties. It can be used as a stabilizer, emulsifier, or suspending agent due to its functional properties and sufficient potential for the use in the pharmaceutical industry **Neeraj Mittal *et al.*, 2016.** Polysaccharide shows the radio-protective effect when polysaccharide combines with selenium, its probability of absorption and its utilization by human body doubles. It acts as organic selenium supplements. With the help of ultrasound-assisted enzymatic extraction, ethanol precipitation, filtration, dialysis, concentration and lyophilisation polysaccharide obtained from black currant was 43.61% shows about 50.90-55.55% s DPPH scavenging activity. Selenized polysaccharide contains selenium of about 171-821μg/g **Changzong Wu *et al.*, 2020**.

Flavanoid was isolated from *L. leucocephala* plant and separated by different techniques like TLC, MS analysis etc. Quercetin-3-O-rhamnoside, caffeic acid, isorhamnetin3-O galactoside etc has been reported by **R. A Hassan *et al.*, 2014** and shows the tremendous antioxidant activity of about 90.31%. Tannin is bitter plant polyphenols either bind and precipitate or shrinks the protein. They help in scavenging the free radical, chelating of transition metals, lipid peroxidation etc. Tanin exhibit microbial activity by complex formation of nucleophilic protein by hydrogen bonding, covalent bonding and nonspecific interaction. They also inactivate microbial enzymes, hinder protein transportation and sometimes inhibit reverse transcription process **Sospeter Ngoci Njeru *et al.*, 2013.**

Plants from Leguminosae family are used for the traditional treatment against depression and anxiety as reported by **L. D. C Kinsou et al., 2019,** The seeds of *L. leucocephala* are a cheap and easy source of proteins with several extracting solvents like water, 5% sodium chloride and 0.5M sodium hydroxide. After extracting and precipitating, mimosine content was relatively less as reported by **Poonam S, Pushpa R Kulkarni 1995.** In recent year it is recommended to have a polysaccharide-rich diet over high-fat diet. Polysaccharide act as upstream signalling for modulating gut microbiota and provide new therapeutic idea **Liqiao Liu *et al.*, 2018**.
Conclusion:. The simple method of soluble polysaccharide extraction was optimized. Seeds of *L.leucocephala* are rich in various phytochemicals which can be explored for a beneficial purpose.

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