ANALYSIS OF ANTIOXIDANTS, PHENOLICS, PROTEIN AND PHYTOCHEMICAL CONSTITUENTS FROM SEEDS OF *Leucaena leucocephala*

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***Abstract*:** *Leucaena leucocephala* is a multipurpose plant, possess various bioactive compounds. Extraction processes play a vital role in optimization. This study aimed to find out maximum extractability of antioxidants, phenolics and protein using different aqueous, organic and their combination from seed. From the above study, the aqueous alkaline solvent is best as compare to other extraction solvents. GC–MS analysis was also carried out with methanol and acetone extraction of seeds to identify various bioactive compounds.

Index Term: Antioxidants, phenolics, protein, bioactive compounds, *Leucaena leucocephala*

1. INTRODUCTION: *Leucaena leucocephala* belongs to family Fabaceae and subfamily Mimosoideae, commonly regarded as river tamarind, horse tamarind or lead tree. Its habitat was previously dominated in Southern Mexico and Central America but now spread across the world. Javanese use young pods as vegetables. People from Indonesia and Thailand use leaves and pods to make various recipes. Its EPPO (European and Mediterranean Plant Protection and Organization) code is LUAGL. According to CABI classification, this plant was listed as invasive in 2018. Features for invasiveness include annual huge seed production, pest resistant and stem propagation. It possesses various bioactive compounds and known for the treatment of severe disease of humans such as cancer and diabetics. Research on natural medicinal agents gained due to cost-effectiveness, cheap availability and very fewer side effects. The seed coat of *L. leucocephala* contains 20% of oil and a good source of commercial gum as reported by **(Vimal and Akhilesh 2013, Meena *et al.*, 2013)**. According to **(Soedarjo *et al.*, 1994)** seed is not suitable for consumption by non-ruminants because of mimosine, a non-protein compound.

L. leucocephala gained interest in research due to resourceful properties. Extraction methods should be appropriate along with standard conditions like type/nature of solvents, pH, temperature etc. for maximum yield. Various extraction processes such as soxhlet extraction, serial exhaustive extraction, maceration, digestion, percolation etc were used by **(Amita and Shalini 2014, Soottawat *et al.*, 2014)**. Extraction of proteins, phenolics from leaves fruits were easy to compare to seeds. Seed coats may hinder the isolation of protein. The present study was designed to explore protein, phenolics and antioxidant activity of various extract of *L. leucocephala* seeds and analyse bioactive compounds using GC-MS.

2. MATERIAL AND METHODS:

2.1 Materials: Dry seeds of *L. leucocephala* (river tamarind), ascorbic acid, acetic acid, acetone, bovine serum albumin, Folin-Ciocalteau reagent, ethanol, ferric chloride, gallic acid, methanol, sodium hydroxide, sodium carbonate, sodium chloride, trichloroacetic acid, tris, potassium ferricyanide, disodium hydrogen phosphate, sodium dihydrogen phosphate and tris.

2.2 Methods:

Preparation of seed sample: Seeds were surface sterilized with $KMnO_4$ and dried. Whole seeds were made into a fine powder and defatted with hexane for 1 hour and stored in an airtight container at 4 ºC for future use **(Shlini and Siddalinga Murthy 2016)**.

Extraction with different aqueous solvents: 5 % extraction (0.5g /10ml) of defatted powdered seed was prepared using aqueous solvents like 50 mM of tris – HCl buffer pH 8.5, acetate buffer pH 5.0, phosphate buffer pH 7.0, different concentration of NaOH (0.05-0.7 M), 0.1M NaCl and distilled water. Extraction was carried out for 30 minutes with constant stirring and centrifuged at 10,000 rpm for 15 minutes at 4°C. Supernatants were made up to 10 ml and used for estimation of antioxidants, phenolics and proteins. Pellets are discarded.

Extraction with different organic solvents: 10 % extraction (1g /10ml) of defatted powdered seed was prepared with 80 % of ethanol, methanol and acetone (v/v). Extraction was carried out for 30 minutes with constant stirring and centrifuged at 10,000 rpm for 15 minutes at. The supernatant was collected and the residue obtained was re-extracted with five volumes of respective solvents. The supernatants were pooled, evaporated to dryness and the residue was dissolved, made up to 25 ml with distilled water. The aqueous solutions were used for the estimation of antioxidants, phenolics and proteins.

Extraction with a combination of alcohol and alkali (NaOH): 10% extraction of defatted powdered seed was prepared using different concentration of ethanol (20-80 %) in 0.6M NaOH for antioxidant and different concentration of methanol (20-80 %) in 0.5M NaOH for phenol and protein. The sample was extracted for 30 minutes with constant stirring and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatants were collected, residue re-extracted with five volumes of respective solvents. Supernatants were pooled and evaporated to dryness. The residue obtained after evaporation was dissolved in distilled water and made volume up to 25 ml with distilled water. The solutions were used for estimation of antioxidants, phenolics and proteins.

2.3 Estimation

Antioxidants: *Reducing power assay*: Total reducing power of the extract was determined according to **(Hinneburg *et al.*, 2006)** with slight modification. The reaction mixture was composed of 0.1 ml of extract, 0.9 ml of water and 0.5 ml of 1% $K_3Fe(CN)_6$, incubated at 50 °C for 20 minutes. To that mixture, 0.5 ml of 10 % TCA was added and centrifuged at 6,000 rpm for 10 minutes. The supernatant was collected and 1 ml of 0.1 % $FeCl_3$ added. Absorbance was measured at 700 nm against blank using UV-visible spectrophotometer. Total antioxidant activity was expressed as ascorbic acid equivalents in g /100g of the dry weight of the sample.

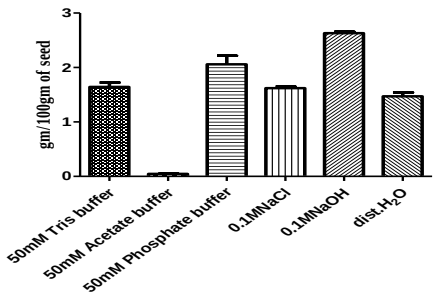
Phenolics: Total soluble phenolics were determined according to **(Shlini and Siddalinga Murthy (2011).** The reaction mixture was composed of 0.1 ml of extract, 7.9 ml of water and 0.5 ml of FC reagent. The tubes were allowed to stand for 3 minutes and 2ml of 20% Na_2CO_3 was added. The resultant mixture was further incubated for 60 minutes. The absorbance was measured at 650 nm against blank using UV-visible spectrophotometer. Total phenolics were expressed as Gallic acid (GAE) equivalent in g /100g of the dry weight of the sample.

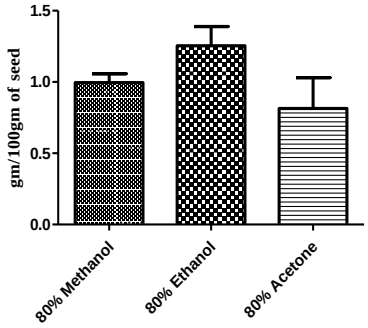
Protein: Estimation of proteins was carried out according to **(Lowry *et al.*, 1951).** Absorbance was measured at 660 nm. Total protein content was expressed as Bovine serum albumin equivalent in g /100 g dry weight of the sample

GC-MS Analysis: Powdered seed was extracted (1g / 10 ml) with two different organic solvents (methanol, acetone) and centrifuged at 10,000 rpm for 15 minutes. Pellet was discarded and 1µl filtrate injected for GC-MS analysis of volatile and semi-volatile bioactive compounds. The column used 30m×250µm. The initial temperature was programmed at 60 °C for 2.8 minutes then was increased to 300°C. The final temperature was held for 6 minutes. The temperature of the injector was 260 °C. Helium was used as a carrier gas **(Mohamed and Benedict 2016).** Interpretation of mass spectra was done using the database from National Institute Standard and Technology (NIST) library having more than 62000 patterns.

3. RESULT AND DISCUSSION:

Estimation of antioxidants: Antioxidants obtained from different solvents shown in Figure 1. Among 5% extraction aqueous solvents, 0.1M NaOH show maximum yield (2.63 g /100g) compare to other aqueous solvents. When the concentration of NaOH increased to 0.6M for extraction yield also increased to 8.195g /100g. A combination of 0.6M NaOH and different percentage of ethanol, antioxidants yield was reduced. With 10 % organic solvent extraction, the yield is negligible.





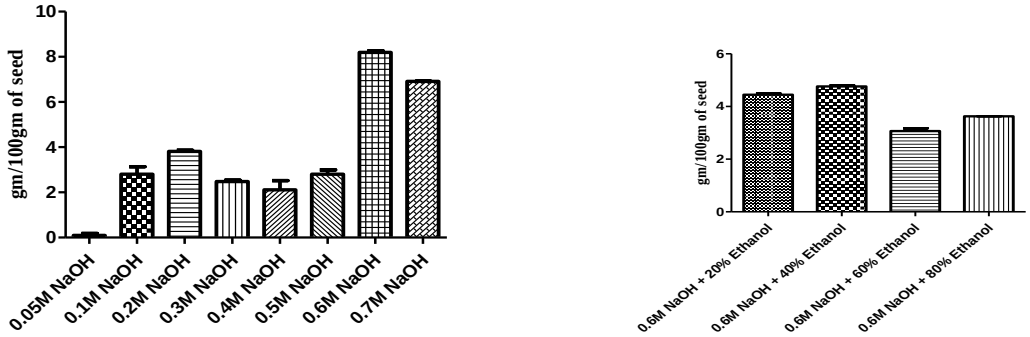


Figure1. Antioxidants obtained using different extraction solvent from *L. leucocephala* seed

Phenolics: Among all aqueous solvent extractions, 0.1M NaOH give the maximum of soluble phenolics (6.45g /100g). When the concentration of NaOH increased to 0.5M yield increased to 9.21g /100g. A combination of 0.5M NaOH in different percentage of methanol yield was 7.09 g /100g. Yield is very less with organic solvent extraction as shown in figure 2.

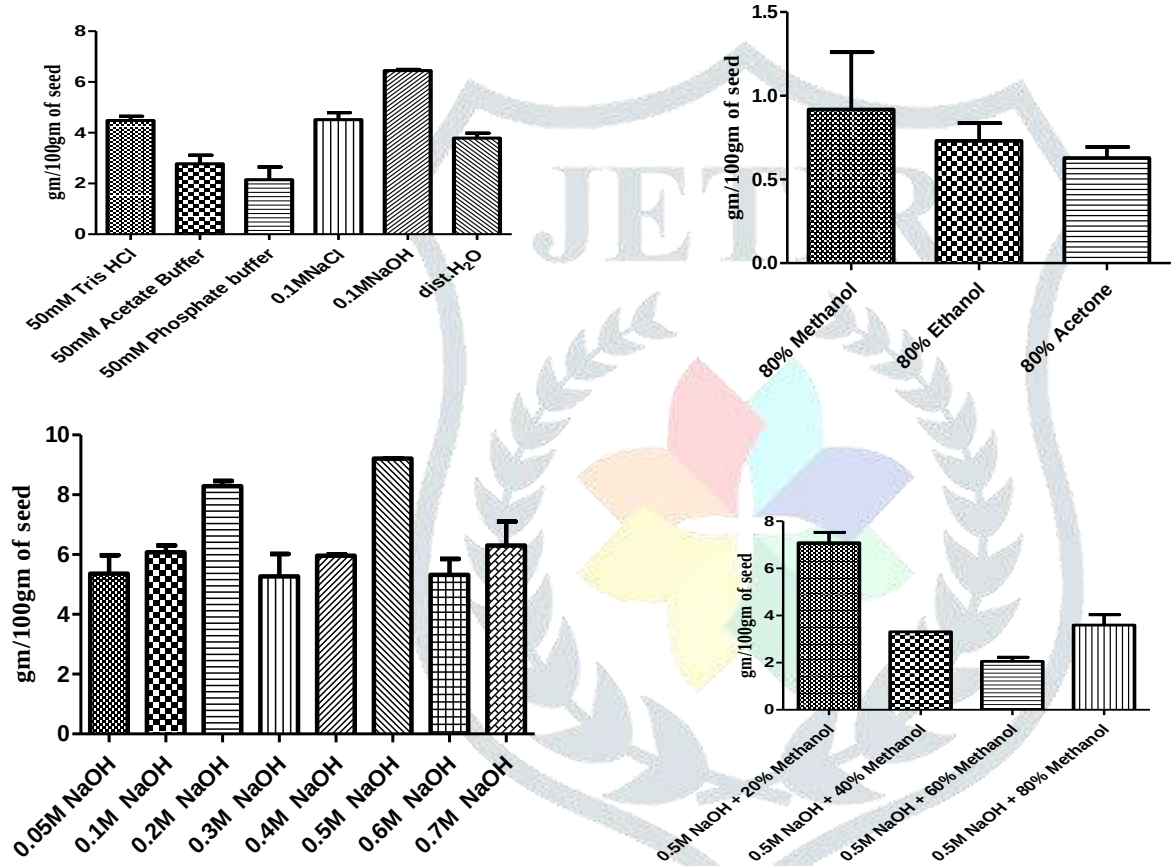


Figure 2: Phenolics obtained using different extraction solvent from *L. leucocephala* seed

Protein: Figure 3 explains protein obtained with various extraction solvents. Among aqueous solvent extraction, 0.1M NaOH yield was 27.63g /100g. With the increase in NaOH concentration to 0.5M, a protein obtained was (43.70g /100g). Among organic solvent extraction, 80% methanol yield was 2.47 g /100g. When aqueous (0.5M NaOH) and different percentage of methanol extraction was combined extraction yield was low.

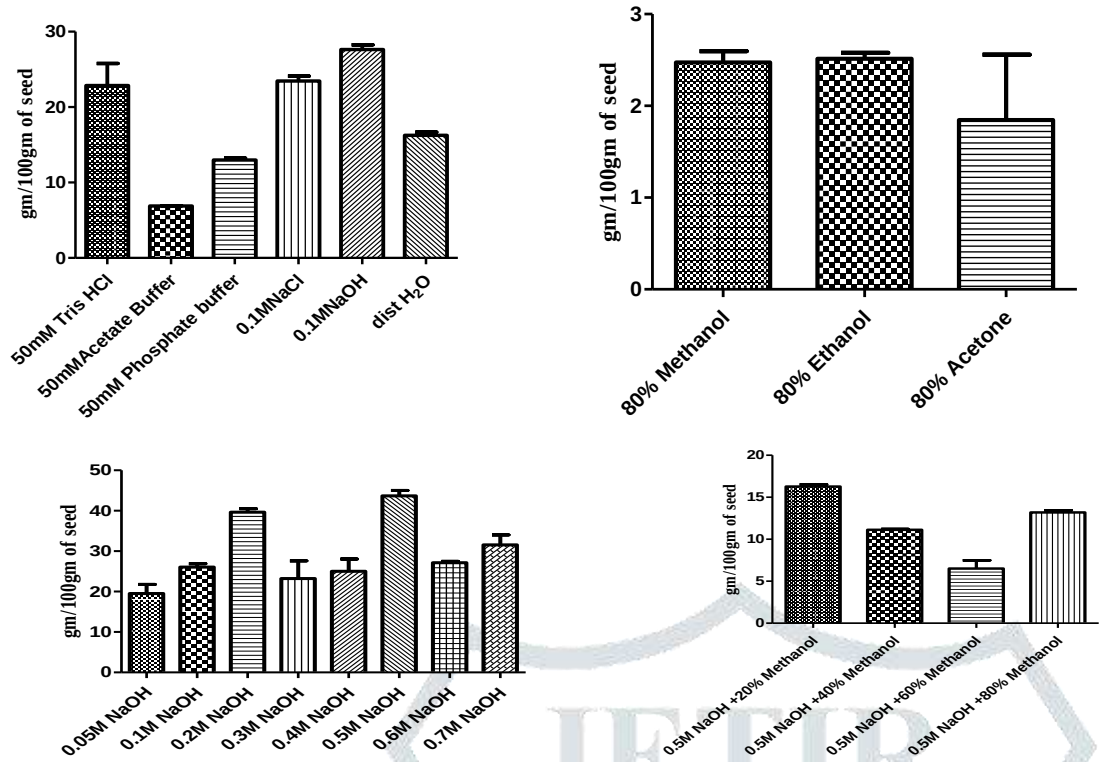


Figure 3: Protein obtained using different extraction solvent from *L. leucocephala* seed.

Thus, from the above results, we can conclude that the alkali solvent is suitable for maximum extractability in comparison with aqueous, organic and their combinations. The organic solvent is not efficient for extraction and efficiency decreases with increase in the concentration of organic solvents.

Bioactive components screening using GC-MS analysis

GC–MS analysis was carried out, a total of 13 peaks with methanol extraction (Figure 4) and 4 peaks were obtained with acetone extraction Figure 5.

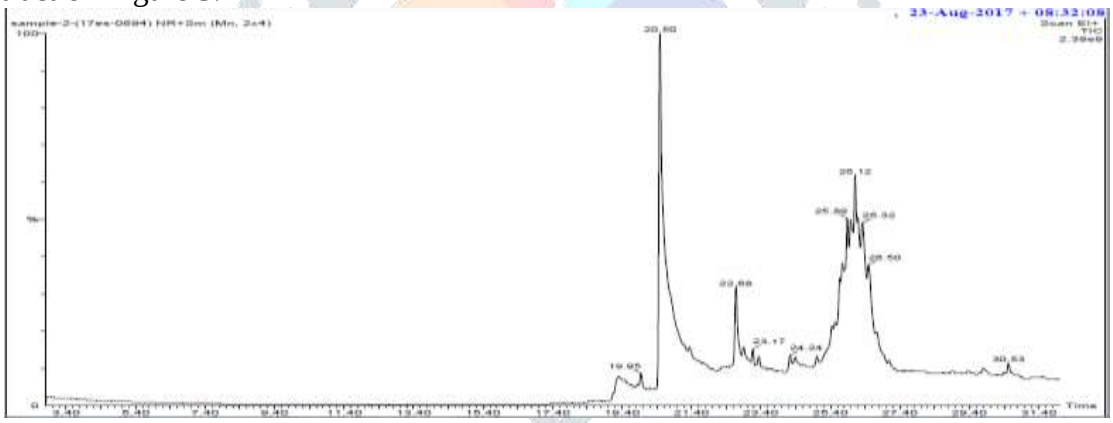


Figure 4 GC-MS chromatogram with methanol extract of *L.leucocephala* seed

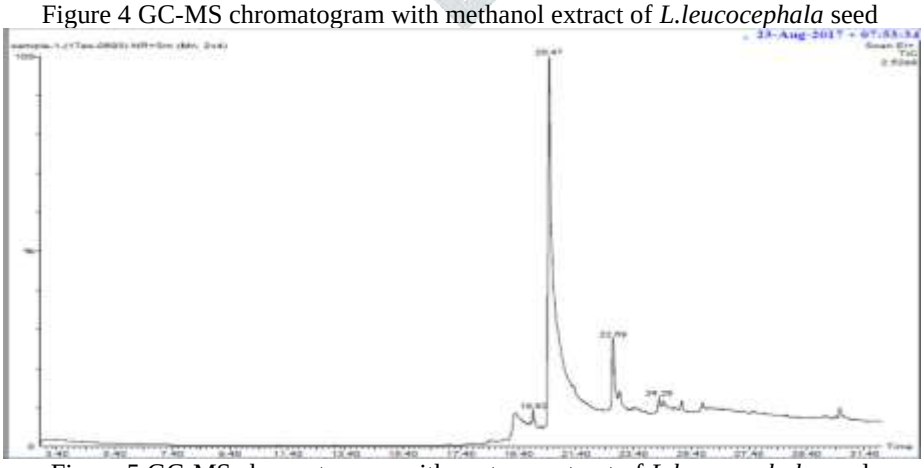


Figure 5 GC-MS chromatogram with acetone extract of *L.leucocephala* seed

Compounds obtained with methanol and acetone extraction are listed in table 1 and 2.

Table 1: List of bioactive compound from methanol extract

No.	Name of the compound	Molecular formula	Molecular weight	Retention Time	Area%
1	1-Hexyl-2-nitrocyclohexane	$C_{12}H_{23}O_2N$	213	20.476	47.828
2	Cis-2,4-dimethylthiane, s,s-dioxide	$C_7H_{14}O_2S$	162	21.341	1.789
3	2-benzene dicarboxylic acid, diisodecyl ester	$C_{28}H_{46}O_4$	446	25.668	1.716
4	Trans-2-methyl-4-n-butylthiane, s,s-dioxide	$C_{10}H_{20}O_2S$	204	25.733	3.127
5	Cyclopentane, 1,2,4-trimethyl	C_8H_{16}	112	25.833	1.829
6	N-butyric acid tetrahydrofurfuryl ester	$C_8H_{14}O_3$	158	25.883	4.275
7	1,2-benzene dicarboxylic acid, diisodecyl ester	$C_{28}H_{46}O_4$	446	25.983	5.066
8	Aziridinone, 1,3-bis(1,1-dimethyl ethyl)	$C_{10}H_{19}ON$	169	26.053	3.380
9	Phthalic acid, 4-fluoro-2-nitrophenyl neopentyl ester	$C_{19}H_{18}O_6NF$	375	26.113	6.310
10	Borane, diethyl(decyloxy)	$C_{14}H_{31}BO$	226	26.193	6.278
11	Sulfurous acid, isohexyl 2-pentyl ester	$C_{11}H_{24}O_3S$	236	26.323	4.901
12	Butanoic acid, 1,1-dimethyl ethyl ester	$C_8H_{16}O_2$	144	26.363	4.233
13	Sulfurous acid, isohexyl 2-pentyl ester	$C_{11}H_{24}O_3S$	236	26.493	5.468

Table 2: List of bioactive compound from acetone extract

No.	Name of the compound	Molecular formula	Molecular weight	Retention Time	Area %
1	Cyclohexanone, 2-ethyl-4-methoxy	$C_9H_{16}O_2$	156	19.265	6.531
2	6-Octadecenoic Acid	$C_{18}H_{34}O_2$	282	20.451	84.306
3	Acetamide, n-2-propynyl	C_5H_7NO	97	21.316	3.044
4	Z-4-nonadecen-1-ol acetate	$C_{21}H_{40}O_2$	324	22.671	6.119

From the result, the yield of 6-Octadecenoic Acid (84.30%) from acetone and 1-Hexyl 2 nitrocyclohexane (47.82%) from methanol extraction was more.

6-Octadecanoic acid (Petro selenic acid) is used for the preparation of oleates, lotions and pharmaceutical solvents, it is classified as omega-12fatty acid. Acetamide is used as a plasticizer and industrial solvents. Z-4-nonadecen-1-ol acetate is also been identified in *Vernonia calvoana* species by **(Iwara A. Iwara *et al.*, 2018).** According to **(Christy Selvamangai and Anusha Bhaskar (2012)**, 1-Hexyl 2 nitro-cyclo-hexane is a ketone compound and has antimicrobial, antioxidants and anti-inflammatory property. Cis-2, 4-dimethylthiane, s,s-dioxide was also reported to present in *Muscodor cinnamon* an endophyte isolated from cinnamon by **(Nakarin Suwannarach *et al.*, 2010)**. Diisodecyl phthalate is an isomeric mixture of phthalates with 10-carbon branched-dialkyl chains, widely used as a plasticizer for polyvinyl chloride **(Kato K *et al.*, 2007)**. Trans-2-methyl-4-n-butylthiane, s,s-dioxide also identified from *Streptomyces pervulas* by **(S Jemiah Naine *et al.*, 2015)** shows cytotoxicity. According to HMDB (Human Metabolome Database) N-butyric acid tetrahydrofurfuryl ester used as flavouring agents with the sweet odour of pineapple or apricot.

Conclusion

Extraction of the defatted seed of *L. leucocephala* with NaOH, antioxidants, phenolics and protein yield was high compare to other aqueous or organic solvent extracts. Seed extraction with methanol provides many bioactive compounds compared to acetone, which is probably attributed to the solvent polarity as shown in GC-MS analysis

The methanol being polar enables the solubility of many polar functional groups of phytochemicals present and this concept will be discussed in future.

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Conflict of interest: No conflict from authors.

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