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DETERMINATION OF TOTAL PHENOLIC, FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY OF *LEUCAENA LEUCOCEPHALA* EXTRACT

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ABSTRACT

Present study was done to evaluate the anti-oxidant and anti-bacterial potential as well as total flavonoid and phenolic content of leaf and pod cover extracts of *L. leucocephala*. Free radical scavenging activity was determined by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay and reducing power by Ferric Reducing Ability of Plasma (FRAP) Assay. Total flavonoid was determined by using Dowd's methods while total phenolic content by Folin-Ciocalteu's method. Anti-bacterial activity activity was investigated through disc diffusion assay. Methanolic and ethanolic leaf extract showed better anti-oxidant and anti-bacterial potential than the pod cover methanol and ethanol extract. Furthermore, there was increased flavonoid and phenolic content in the leaf extract. Thus, we infer that due to the presence of secondary metabolites (phenol and flavonoids) in the leaf extract, it can serve as a potential source of natural anti-oxidant and anti-bacterial agent.

KEYWORDS: *Leucaena leucocephala*, Anti-bacterial, Anti-oxidant, Flavonoid, Phenolic.

INTRODUCTION

In our body, highly reactive free radicals such as hydroxy radical, superoxide anion and singlet oxygen are generated during normal metabolism. Generally, these free radicals are neutralized by endogenous anti-oxidants. When there is increased production of free radicals, there is depletion of anti-oxidants.[1] These free radicals then cause damage to lipids, proteins and DNA leading to various diseases such as myocardial infarction, asthma, diabetes,

neurodegenerative disease and cancer etc.[2] Thus, anti-oxidants are required either to neutralize or to scavenge these free radicals.

Since ancient times, medicinal plants have been used to prevent or treat diseases. Various studies have established their role as natural anti-oxidants.[3,4] Even in modern system of medicine, people prefer to use plant products as they are easily accessible, less costly and are safer than synthetic compounds. Plants are rich source of various secondary metabolites such as phenol, flavonoids, alkaloids, glycosides, tannins etc. Studies have shown the phenol and flavonoids present in different plant parts have protected against diseases caused by oxidative stress.[5,6]

Leucaena leucocephala (*L. leucocephala*; Family: Fabaceae) is a small tree or shrub, commonly known as Kubabul in India. It is native to southern Maxico and northern central America and distributed to Asia, South America, Europe, Africa and areas with warm climate.[7] It is mainly used a livestock fodder due to high protein content.[8] In various countries, pods and leaves of *Leucaena* are consumed by human as vegetables, soup and salads.[9] It is used as antihelminitic, anti-diabetic, abortifacient, anti-proliferative, anticancer, anti-inflammatory and anti-oxidant.[10] Experimental studies have demonstrated anti-oxidant and anti-diabetic activities of seed and leaf extract.[11,12] Previous studies have reported the antihelmintic and cytotoxic activities of *L. leucocephala* pod cover extract.[13-15]

As this plant possess various medicinal properties so, in this study, we evaluated the anti-bacterial and anti-oxidant potential of leaf and pod cover extracts of *L. leucocephala*.

MATERIALS AND METHODS

Reagents

Antibiotics (Ampicillin and chloramphenicol) discs were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used in the experiment were of analytical grade and procured from Sigma Aldrich, USA.

Plant material collection and extraction

L. leucocephala leaves and pod covers were collected from the Sri Venkateswara College, University of Delhi, New Delhi, India. After collection, plant parts were washed thoroughly with water and dried in shade at room temperature (RT). Dried plant material was grounded and then passed through 1 mm sieve. Sieved powder (10 gm) was dissolved in methanol &

ethanol (100 ml) respectively and stirred overnight on magnetic stirrer (200 rpm). After that, it was passed through Whatman® no. 41 filter paper. Filtrate was dried with rotary evaporator (Labcone Digital rotary evaporator, EW-28630-10) at 40°C and stored at −20°C till further use.

Determination of anti-bacterial activity

Bacterial culture

Anti-bacterial activity of plant extracts were estimated using freshwater bacterial [*Aeromonas hydrophila* (MTCC 1739), *Escherichia coli* (MTCC 1575), *Enterococcus faecalis* (MTCC 2729), *Pseudomonas aeruginosa* (MTCC 1034), *Staphylococcus aureus* (MTCC 3160)] and marine water bacteria [*Vibrio anguillarum* (kind gift from Debra L. Milton, Professor, Department of Molecular Biology, Umea University, Umea, Sweden) and *Vibrio harveyi* (MTCC 7954)]. Bacteria were inoculated in conical flasks containing 50 ml Luria-Bertani culture media (pH 7.4) containing 1% or 2% NaCl (for freshwater bacteria and marine water bacteria respectively). The inoculated bacterial flasks were allowed to grow overnight at 37°C with gentle orbital shaking (200 rpm).

Disc diffusion assay (DDA)

For DDA, standard inoculum of $1x10^8$ was prepared in 1.5% sterilized agar plates with 1% NaCl for fresh water bacteria and with 2% NaCl for marine water bacteria. Sterile circular paper discs (thickness 1 mm; diameter 6 mm) were impregnated with 20 µl plant extract (200 100 and 50 µg per disc) prepared in 0.2% DMSO and placed on agar plates. DMSO (0.2%) and antibiotics [Ampicillin (10 µg per disc), Chloramphenicol (30 µg per disc)] were used as negative and positive control respectively.

Determination of anti-oxidant activity

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

Anti-oxidant activity of extracts was determined using DPPH radical scavenging method [Brand-Williams *et al*. (1995) modified by Miliauskas *et al*. (2004)].[16,17] 300 µl of methanolic solution of DPPH (6×10^{-5} M) was added to 10 µl of freshly prepared respective extract (0.5 mg/ml) in a 96 well microtiter plate. Plate was incubated at 37°C for 20 min. and absorbance was measured at 515 nm. Methanol/ethanol solutions were used as respective control. Free radical scavenging activity of plant extract was calculated as percentage inhibition using standard formula $[(A_B-A_S)/A_B] \times 100$; where A_B was absorbance of blank

and As was absorbance of sample.[18,19] Samples were processed in quadruplicates in this assay.

Ferric Reducing Ability of Plasma (FRAP) Assay

Reducing ability of extracts were determined using FRAP assay. To the 10 µl of respective extract solution (0.5 mg/ml), 30 µl of distilled water and 300 µl of freshly prepared FRAP solution [containing 10 parts of 300 mM acetate buffer (pH 3.6), 1 part of 10 mM TPTZ (2,4,6-tripyridyl triazine) in 40 mM HCl and 1 part of 20 mM ferric chloride] was added and incubated at 37°C for 30 min. Acetate buffer was used as control. A standard graph was plotted by serial double dilution of ferrous sulphate (20.0 to 0.009 mg/ml) as substrate. Absorbance was taken at 593 nm and reducing activity of extract is represented as millimoles of Fe^{2+}/mg of the extract.

Measurement of Total Phenolic Content (TPC)

The Folin-Ciocalteu's method was used for measurement of phenolic content in extracts.[20] 0.5 ml Folin-Ciocalteu's Phenol reagent and 1.5 ml distilled water was added to 1 ml of extract (2 mg/ml) followed by addition of 20% sodium carbonate solution (1.25 ml). Solution was incubated for 2 hrs in dark at 25°C with intermittent shaking and absorbance was taken at 760 nm. A standard graph was plotted using serial double dilutions of gallic acid (20-0.5 µg/ml).[19] Total phenolic content is expressed as the µg of gallic acid equivalent flavonoid present per mg of extract.

Measurement of Total Flavonoids

Dowd method was used for measurement of total flavonoids in both the extracts of leaf and pod cover.[21] 1 ml of 2% Aluminum tri-chloride solution (prepared in methanol) was added to 1 ml of plant extract (10 mg/ml) at RT. Absorbance was recorded at 415 nm with methanol/ethanol solution as control (carrier blanks).[19] Flavonoid concentration was calculated using serial double dilution of standard quercetin (8.33-0.032 mg/ml) and expressed in µg of quercetin equivalent flavonoids present per mg of the extract.

Statistical analysis

All values are represented as mean ± SD. Data were analysed by Students t test and followed by ANOVA using SigmaPlot 12.0 software. P value less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Anti-bacterial activity of L. leucocephala extract

Table 1 represents the anti-bacterial activity of *L. leucocephala* leaf and pod cover extracts. Methanol extract of leaf showed maximum sensitive against *A. hydrophila* (gram negative) and ethanol extract was against *E. faecalis* (gram positive) bacteria. On the other hand, both the leaf extracts shown least activity against *V. harveyi* (gram negative) bacteria. Pod cover methanol and ethanol extract was maximum sensitive against *S. aureus* (gram positive) and *V. harveyi* (gram negative) bacteria respectively at 200 µg concentration whereas at other two maximum against *A. hydrophila* and *V. anguillarum* (-) (gram negative) bacteria respectively. Previously, Mohammed et al., 2015 reported the anti-bacterial activity of *L. leucocephala* leaf extract.[22]

Anti-oxidant activity of L. leucocephala extract

In this study, anti-oxidant potential of the extracts was estimated with DPPH and FRAP assay. In DPPH assay, change in colour of DPPH solution depends on the ability of substance to donate hydrogen atom or due to its radical scavenging capacity. When an anti-oxidant added to DPPH solution, anti-oxidant changes its colour from violet (radical form) to colourless (non-radical form) by donating a hydrogen atom. Pod cover methanol extract showed anti-oxidant activity equivalent to the leaf methanol extract. However, leaf ethanol extract shown significantly higher anti-oxidant activity than pod cover ethanol extract ($P<0.05$). Both methanol extracts demonstrated significantly higher anti-oxidant activity than their respective ethanol extract (Figure 1). FRAP assay indicates reducing ability of the substance. Both leaf extract showed significantly ($P<0.05$) higher anti-oxidant activity than their respective ethanol extracts (Figure 2). Thus, in both the assays, leaf extracts have shown more anti-oxidant activity in comparison to pod cover extract. These results are supported by the previous studies who reported the anti-oxidant activity of leaf extract.[22,23]

Total flavonoid and phenolic content of L. leucocephala extract

Table 2 indicates presence of total flavonoid and phenolic content in the leaf and pod cover extracts. Methanol and ethanol extracts of leaf extract contained more flavonoid content than other extracts ($P<0.05$). Similarly, Phenolic contents were significantly higher in leaf methanolic and ethanolic extract than pod cover extracts ($P<0.05$). Previous studies suggest that anti-oxidant activity of plant could be due to presence of secondary metabolites in different plant parts.[24] Flavonoids and phenols act as radical scavenger due to presence of

hydroxyl group in their structure.[25] Accordingly, in our study, anti-oxidant activity of leaf extract correlates with the increased flavonoid and phenol content in the extract. Previous study suggested that the presence of phenolic and flavonoid compounds in the *L. leucocecphala* leaf was responsible for its anti-oxidant potential.[26]

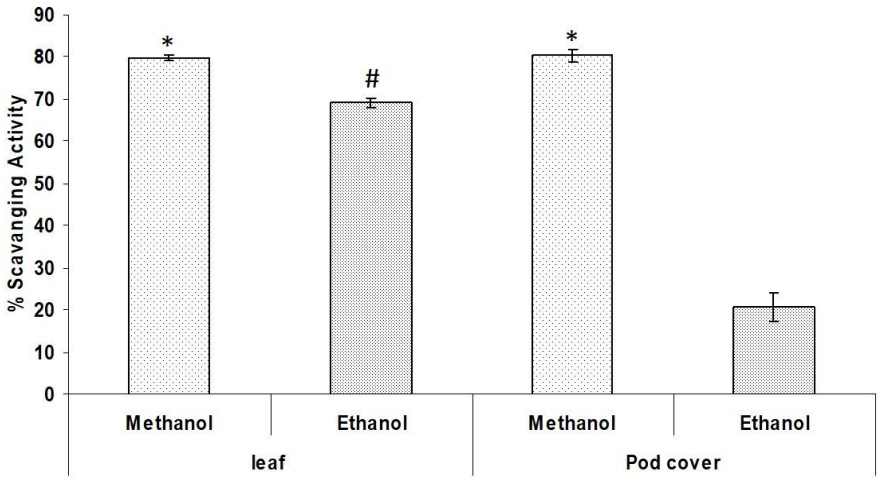

Figure 1: Anti-oxidant activity (DPPH assay) of *L. Leucocephala* leaf and pod cover extracts. [*significant difference in ethanolic and methanolic extract of same plant part; #significant difference in within the same extract of different plant part (leaf vs pod cover)]

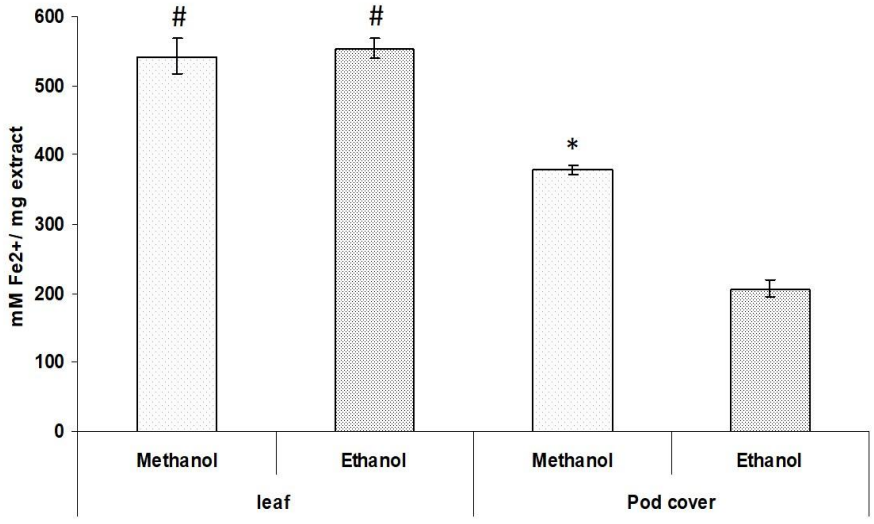

Figure 2: Anti-oxidant activity (FRAP assay) of *L. Leucocephala* leaf and pod cover extracts. [*significant difference in ethanolic and methanolic extract of same plant part; #significant difference in within the same extract of different plant part (leaf vs pod cover)]

Table 1: Anti-bacterial activity of *L. leucocephala* leaf and pod cover extracts.

Plant Part extract		Bacterial Strain	Diameter of Zone of Inhibition (in mm)			Standard Antibiotics	
			200µl/disc	100µl/disc	50µl/disc	Ampicillin	Chloramphenicol
L. leucocephala Leaf	Methanol	*Aeromonas hydrophila* (-)	12.67 ± 0.33	11.33 ± 0.33*	9.90 ± 0.21	6	9
		Escherichia coli (-)	7.33 ± 0.17	7.17 ± 0.17	6.83 ± 0.17	14	22
		Enterococcus faecalis (+)	11.33 ± 0.33#	9.17 ± 0.17#	8.17 ± 0.17#	6	27
		Pseudomonas aeruginosa (-)	11.33 ± 0.33#	9.33 ± 0.33#*	7.83 ± 0.17	22	27
		Staphylococcus aureus (+)	11.17 ± 0.17	9.67 ± 0.33#	8.33 ± 0.17	6	28
		Vibrio anguillarum (-)	8.17 ± 0.17	7.33 ± 0.17	6.67 ± 0.17	11	24
		Vibrio harveyi (-)	7.83 ± 0.17	7.17 ± 0.17	6.60 ± 0.10	7	26
	Ethanol	*Aeromonas hydrophila* (-)	12.5 ± 0.29	9.83 ± 0.17	8.67 ± 0.17*	6	9
		Escherichia coli (-)	11.5 ± 0.29#*	9.67 ± 0.17#*	8.67 ± 0.17#*	14	22
		Enterococcus faecalis (+)	14.17 ± 0.17#*	12.83 ± 0.17#*	10.83 ± 0.17*	6	27
		Pseudomonas aeruginosa (-)	12.5 ± 0.29#*	8.50 ± 0.29#*	8.17 ± 0.17#	22	27
		Staphylococcus aureus (+)	11.67 ± 0.33	10.17 ± 0.17	8.83 ± 0.17*	6	28
		Vibrio anguillarum (-)	12.33 ± 0.33*	9.83 ± 0.17*	7.83 ± 0.17*	11	24
		Vibrio harveyi (-)	8.93 ± 0.07#*	7.83 ± 0.17	6.83 ± 0.17	7	26
L. leucocephala Pod Cover	Methanol	*Aeromonas hydrophila* (-)	12.25 ± 0.33	11.00 ± 0.17*	9.90 ± 0.33	6	9
		Escherichia coli (-)	10.33 ± 0.17#*	9.83 ± 0.17#*	8.83 ± 0.17#*	14	22
		Enterococcus faecalis (+)	8.17 ± 0.33	7.83 ± 0.17	7.67 ± 0.17	6	27
		Pseudomonas aeruginosa (-)	9.33 ± 0.17	8.17 ± 0.17*	7.33 ± 0.17	22	27
		Staphylococcus aureus (+)	12.67 ± 0.33#*	8.67 ± 0.33	8.17 ± 0.17	6	28
		Vibrio anguillarum (-)	11.83 ± 0.17#	10.33 ± 0.33#	8.50 ± 0.29#	11	24
		Vibrio harveyi (-)	10.33 ± 0.33#	8.83 ± 0.17#	8.17 ± 0.17#	7	26
	Ethanol	*Aeromonas hydrophila* (-)	12.75 ± 0.33	9.67 ± 0.17	8.83 ± 0.33	6	9
		Escherichia coli (-)	9.83 ± 0.17	8.40 ± 0.21	7.93 ± 0.07	14	22

		***Enterococcus faecalis* (+)**	12.33 ± 0.17*	11.33 ± 0.17*	11.17 ± 0.17#*	6	27
		***Pseudomonas aeruginosa* (-)**	9.17 ± 0.17	7.33 ± 0.17	7.17 ± 0.17	22	27
		***Staphylococcus aureus* (+)**	11.67 ± 0.33	9.67 ± 0.33*	8.33 ± 0.33	6	28
		***Vibrio anguillarum* (-)**	12.67 ± 0.67*	11.67 ± 0.33#*	10.33 ± 0.33#*	11	24
		***Vibrio harveyi* (-)**	14.33 ± 0.33*	11.33 ± 0.33#*	9.33 ± 0.17#*	7	26

*significant difference in ethanolic and methanolic extract at same concentration of same plant part; #significant difference in within the same extract of different plant part (leaf vs pod cover)

Table 2: Total flavonoid and phenolic content of *L. leucocephala* leaf and pod cover extracts.

Extract	Total Flavonoid content	Total Phenolic content
Leaf		
Methanol extract	38.19 ± 2.91*#	57.40 ± 4.79#
Ethanol extract	34.46 ± 0.44#	69.05 ± 1.98*#
Pod cover		
Methanol extract	5.08 ± 0.93	26.96 ± 1.16*
Ethanol extract	7.28 ± 0.78*	13.98 ± 0.35

*significant difference in ethanolic and methanolic extract of same plant part; #significant difference in within the same extract of different plant part (leaf vs pod cover)

CONCLUSION

Based on the results of present study, it can be concluded that *L. leucocephala* leaf extract has higher anti-oxidant and anti-bacterial activities than the pod cover extract. These properties can be attributed to presence of total flavonoid and phenolic compounds or other phytochemicals in the leaf extract. Further in vivo studies are suggested to confirm their anti-oxidant and anti-bacterial potential.

CONFLICTS OF INTEREST

Authors declare that there is no conflict of interest.

REFERENCES

1. Ye ZW, Zhang J, Townsend DM, Tew KD. Oxidative stress, redox regulation and diseases of cellular differentiation. Biochimica et Biophysica Acta, 2015; 1850: 1607–1621.

2. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology, 2007; 39: 44–84.
3. Lalhminghlui K, Jagetia GC. Evaluation of the free-radical scavenging and antioxidant activities of Chilauni, Schima wallichii Korth in vitro. Future Science OA, 2018; 4: FSO272.
4. Johari MA, Khong HY. Total Phenolic Content and Antioxidant and Antibacterial Activities of *Pereskia bleo*. Advances in Pharmacological Sciences, 2019; 2019: 7428593.
5. Tahmina Monowar, Md. Sayedur Rahman, Subhash J. Bhore, Gunasunderi Raju, Kathiresan V. Sathasivam. Secondary Metabolites Profiling of Acinetobacter baumannii Associated with Chili (Capsicum annuum L.) Leaves and Concentration Dependent Antioxidant and Prooxidant Properties. Biomed Research International, 2019; 2019: 6951927.
6. Ramya R, M Kalaiselvi, R Narmadha, D Gomathi, V Bhuvaneshwari, R Amsaveni, K Devaki. Secondary metabolite credentials and in vitro free radical scavenging activity of Alpinia calcarata. Journal of Acute Medicine, 2015; 5: 33-37.
7. Brewbaker JL, Sorensson CT. New tree crops from interspecific Leucaena hybrids. In: Janick, J. and Simon, J.E. (eds), Advances in New Crops. Timber Press Portland, 1990; 283-289.
8. Nirmal NP, Benjakul S. Inhibition of melanosis formation in Pacific white shrimp by the extract of lead (*Leucaena leucocephala*) seed. Food Chemistry, 2011; 128: 427-432.
9. Meena Devi VN, Ariharan VN, Nagendra Prasad P. Nutritive Value and Potential Uses of Leucaena Leucocephala as Biofuel – A Mini Review. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 2013; 4: 515-521.
10. Zayed M, Benedict S. Phytochemical constituents of the leaves of Leucaena leucocephala from malaysia. International Journal of Pharmacy and Pharmaceutical Sciences, 2016; 8: 174-179.
11. Chowtivannakul S, Talubmook C. Antioxidant and antidiabetic activities of leaf and seed extracts from Leucaena leucocephala (Lam.) de Wit. Proceeding of NATPRO 4. Chiang Mai, Thailand, 2012; 356-359.
12. Chowtivannakul P, Srichaikul B, Talubmook C. Antidiabetic and antioxidant activities of seed extract from Leucaenaleucocephala (Lam.) de Wit. Agricultural and Natural Resources, 2016; 50: 357-361.

13. Ibrahim MT. Cytotoxic activities of flavonoid glycosides isolated from Leucaena leucocephala pods cultivated in Egypt. Journal of Pharmacy Research, 2017; 11(2): 108-115.
14. She LC, Liu CM, Chen CT, Li HT, Li WJ, Chen CY. The anti-cancer and anti-metastasis effects of phytochemical constituents from Leucaena leucocephala. Biomedical Research, 2017; 28(7): 2893-2897.
15. Rivero-Perez N, Jaramillo CA, Peláez-Acero A, et al. Anthelmintic activity of Leucaena leucocephala pod on gastrointestinal nematodes of sheep (in vitro). Abanico Veterinario 2019; 9(1): 1-9.
16. Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. LWT- Food Science and Technology, 1995; 28: 25-30.
17. Miliauskas G, Venskutonis PR, Van Beek TA. Screening of radical scavenging activity of some medicinal plants and aromatic plant extract. Food Chemistry, 2004; 85: 231-237.
18. Hatano T, Kagawa H, Yasuhara T, Okuda T. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Chemical and Pharmaceutical Bulletin, 1988; 36: 2090-97.
19. Sarikurkcu C, Ozer MS, Cakir A, Eskici M, Mete E. GC/MS Evaluation and *In Vitro* Antioxidant Activity of Essential Oil and Solvent Extracts of an Endemic Plant Used as Folk Remedy in Turkey: *Phlomis bourgaei Boiss*. Evidence Based Complementary and Alternative Medicine, 2013; 2013: 1-7.
20. Djeridane A, Yousfi M, Nadjemi B, Boutassouna D, Stocker P, Vidal N. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chemistry, 2006; 97: 654-60.
21. Arvouet-Grand A, Vennet B, Pourrat A, Legret P. Standardization of a propolis extract and identification of the main constituents. Journal de pharmacie de Belgique, 1994; 49: 462-68.
22. Mohammed RS, Souda SSE, Taie HAA, Moharam ME, Shaker KH. Antioxidant, antimicrobial activities of flavonoids glycoside from Leucaena leucocephala leaves. Journal of Applied Pharmaceutical Science, 2015; 5: 138-147.
23. Aderogba MA, McGawc LJ, Bezabihb BT, Abegazb BM. Antioxidant activity and cytotoxicity study of leucaena leucocephala (lam.) De wit leaf extract constituents. Nigerian Journal of Natural Products and Medicine, 2009; 13: 65-68.
24. Cao G, Sofic E, Prior RL. Antioxidant and pro-oxidant behaviour of flavonoids: Structure-activity relationships. FreeRad. Biol. Med., 1997; 22: 749-760.

25. RiceEvans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Rad Res., 1995; 22: 375-383.
26. Sharma P, Chaurasia S. Evaluation of Total Phenolic, Flavonoid Contents and Antioxidant Activity of Acokanthera oppositifolia and Leucaena leucocephala. International Journal of Pharmacognosy and Phytochemical Research, 2014-15; 7: 175-180.