Available online at www.sciencedirect.com

ScienceDirect

Procedia Chemistry 16 (2015) 164 – 170

Procedia Chemistry

International Symposium on Applied Chemistry 2015 (ISAC 2015)

Extraction and Quantification of Toxic Compound Mimosine from *Leucaena leucocephala* Leaves

Zul Ilham*, Hanisjasymah Hamidon, Nurul Amalina Rosji, Norfadhilah Ramli, Normaniza Osman

Institute of Biological Sciences, Faculty of Science, University of Malaya 50603 Kuala Lumpur, Malaysia

Abstract

The existence of mimosine in *Leucaena leucocephala* prevents the utilization of its biomass residues (after energy conversion) as animal feed. In this study, mimosine quantification in *Leucaena leucocephala* was carried out by using rapid colorimetric method. In addition, two different extraction methods which are soxhlet extraction with either distilled water or ethyl acetate as extraction solvent and digestion method were used to compare its efficiency in extracting the mimosine from *Leucaena leucocephala* leaves. The samples from both extraction methods were then clarified by boiling it with 30 mg activated carbon and filtered. The absorbance of diluted aliquot of 1-2-3-4-5 mL was then obtained from spectrophotometer at the wavelength of 535 nm. The concentration of mimosine from soxhlet extraction with distilled water was found to be higher compared to the digestion method but in contrast with ethyl acetate solvent extraction.

Peer-review under responsibility of Research Center for Chemistry, Indonesian Institute of Sciences

Keywords: Mimosine; Digestion; Soxhlet; *Leucaena leucocephala*.

1. Introduction

Leucaena leucocephala was first recognized in Central America and the Yucatan Peninsula of Mexico by Spanish adventurer who carried Leucaena feed and seed on their galleons to the Philippines to feed their stock. It has spread to most countries of the tropical world since then where it was used as shade plants for their crops. The genus Leucaena has over 50 names ascribe to it such as while leadtree in English, *subabul* in India, *lamtoro gung* in

* Corresponding author. Tel.: 03-79674014; fax: +03-79674178.
E-mail address: ilham@um.edu.my

Peer-review under responsibility of Research Center for Chemistry, Indonesian Institute of Sciences
doi:10.1016/j.proche.2015.12.029

Indonesia, giant *ipil-ipil* in Philippines and also known as *petai belalang* in Malaysia. Apart from that, Leucaena also can provide firewood, timber, human food, green manure and act as erosion control[1]. Potential erosion control plant study was carried out that showed *Leucaena leucocephala* displays the characters of extensive, dense rooting and deep penetration[2]. These characters is important for an erosion control plant because not only it can prevent surficial erosion but deep-seated erosion as well. *Leucaena leucocephala* seeds was also found to have medicinal values of anthelmintic (capabilities of destroying parasitic worms), antidiabetic and has a broad spectrum antibacterial activity[3].

Mimosine or sometimes referred as Leucaenine or Leucaenol is a non-protein amino acid was first discovered by J. Renz in 1936 that he isolated from the sap of young shoots and leaves of *Mimosa pudica*[4,5]. Leucaena contains toxic amino acid mimosine which may reach 12% of the dry matter in growing tips such as foliage and pods but less in dry matter in young leaves for about 3-5%[6]. Mimosine content in Leucaena has the potential as bio-herbicide as it has the phytotoxic effects on various plants. In spite of that, mimosine exudation in Leucaena from soaking through water and area which have a great annual rainfall often suffered soil injury because of the alleleophatic feature[7]. Mimosine and its degradation product 3-hydroxy-4(1H)-pyridone (3, 4-DHP) are both toxic when ingested by both ruminants and non-ruminants. Although some ruminants contains bacteria (*Synergistes jonesii*) in their rumen that degrades mimosine and DHP and protect them against its harmful effect[8], in animals generally, mimosine caused ill health and growth reduction when Leucaena leaves was fed to their diet[6] and its degradation product, DHP causes goiter, loss of hair and reduced productivity[9]. The loss of hair observed was caused by inhibition of conversion of methionine to cysteine, an important component of hair[10]. Leucaena have high total crude protein content in leaves for about 26.14% and in seeds for about 33.44%[11] making it suitable as feed source for animal but the high content of mimosine in *Leucaena leucocephala* plants despite its high protein content prevent further usage of it as animal feed.

Thus, this research was carried out to identify the presence of toxic compound mimosine in *Leucaena leucocephala* foliage using different types of methods and solvents. Other than that, this research also highlighted the efficient way to reduce the toxic compound mimosine so the usage of the plant can be maximise either as livestock fodder, as an option for energy sources.

2. Material and Methods

2.1 Materials

The *Leucaena leucocephala* leaves used in this study was collected from a controlled area in University of Malaya, Malaysia (Glami Lemi Biotechnology Research Center). Pure L-Mimosine from Koa hoale seeds was obtained from Sigma-Aldrich. The hydrocloric acid of 37%, Iron (III) chloride hexahydrate (FeCl3), and ethyl acetate was purchased from Friendemann Schmidt Chemicals. The equipment used were TX3202L Top loading Balance, Shimadzu, United States, IKA® C-MAG HS 10 IKAMAG hot plate, Syringe Filter with Sartorius 0.2µm for purification of activated charcoal and Jenway 7305 Series Spectrophotometer for determination of absorbance of mimosine.

2.2 Experimental procedure

The leaves was removed from its stem and was let dry at room temperature for about two days. After two days, the dried leaves was blend until fine powder size was achieved. The powder will then be treated by using two different extraction methods before quantifying its concentration. Extracts from soxhlet extraction was obtained from reacting 40 g of dried *Leucaena leucocephala* leaves with 400 mL of distilled water as the solvent. The cycles was ensured to be constant before it was let to run for 8 hours at 350 °C. The extract along with the solid was transferred into 250 mL Schott Duran® storage bottle and was allowed to stand until most of the solids get settled to the bottom. The soxhlet extraction process was also repeated for two times and by using another solvent, ethyl acetate.

HCl digestion was conducted by weighing 40 g of dried *Leucaena leucocephala* leaves and was placed in an 800 mL beaker. Volume of 400 mL of 0.1N HCl was added and the mixture was boiled at the temperature of 85 °C on

hot plate for an hour. The mixture was transferred into 250 mL Schott Duran® storage bottle and was allowed to stand until most of the solids get settled to the bottom. The HCl digestion process was repeated for two times.

2.3 Preparation of standard and calibration curve

Exactly 0.025 g of L-Mimosine from Koa hoale seeds was dissolved in a 50 mL conical flask with 25 mL 0.1N HCl. A 0.1N HCl was produced by diluting 9.9 mL of 37% HCl with 990.1 mL distilled water inside 1 L conical flask. Aliquot of 0(blank)-1-2-3-4-5 mL of the liquid were transferred into 6 different conical flask and each were added with 10 mL of 0.1N HCl and 4 mL of 0.5% $FeCl_3$ in 0.1N HCl. The 0.5% $FeCl_3$ in 0.1N HCl was obtained from dilution of 0.5 g of FeCl3 with 100 mL of 0.1N HCl inside 250 mL conical flask. The final volume of 6 different aliquots were raised until 100 mL with distilled water. The absorbance of each diluted aliquots were determined and a graph of absorbance against concentration was plotted. The concentration of mimosine from the reaction mixture were 0(blank)-10-20-30-40-50 µg/mL. The steps were repeated with alteration that this time only 1-3-5 mL of the aliquots was tested with Jenway 7305 Series Spectrophotometer.

2.4 Clarification of extracts

A volume of 10 mL of supernatant liquid from the extract was transferred in a 150 mL beaker containing 0.03 g activated carbon. Distilled water was then added until the volume reached about 25 mL. The beaker was covered with an aluminum foil and the liquid was boiled for 15 minutes on a hot plate. The liquid was then filtered by using syringe filter and was placed in a 50 mL conical flask. The steps were repeated two times for both extraction methods.

2.5 Mimosine quantification

Mimosine quantification in *Leucaena leucocephala* leaves was determined by using rapid colorimetric method[12] with slight modification of the ratio of the sample and solvent of the extracts. First, 0.1N HCl was added to the filtrate of clarified extract until the volume became 50 mL. Aliquot of 1-2-3-4-5 mL from the filtrate was transferred into 5 different 100 mL conical flasks and each were labelled as B, C, D, E and F. Next, each of the labelled flasks were added with 10 mL of 0.1N HCl and 4 mL of 0.5% $FeCl_3$ in 0.1N HCl. The volume was made up to 100 mL by adding distilled water. The absorbance of each diluted aliquots were read at the wavelength of 535 nm. The steps were repeated two times for each extraction methods and the concentration of mimosine was then determined by using calibration curve prepared previously.

3. Results and Discussion

3.1 Qualitative analysis

Mimosine and its derivative (DHP) could be identified with observing the colour changes when acidified $FeCl_3$ was added. Figure 1 showing that there are differences between reaction mixture of L-mimosine from Koa hoale seeds and extracts obtained from extraction methods. The colour changes observed from the experiment of goat's urine when Leucaena leaves was being fed is purple colour development[13]. Since mimosine is one of the phenol compound, addition of $FeCl_3$ may results in red, blue, green or purple coloration. In addition, addition of $FeCl_3$ to a solution of mimosine in water gave a red coloration, which changed to violet on adding more $FeCl_3$[14]. L-mimosine reaction mixture exhibit violet coloration compared to extracts reaction mixture which gave results to dark purple coloration. The difference in colour presumably because of the existence of DHP since L-mimosine from Koa hoale seed was purely mimosine. A study stated that 3, 4-DHP colour reaction with acidified $FeCl_3$ was purple and 2, 3-DHP reaction mixture colour was observed to be blue[15]. The dissimilarity of the colour of mimosine and DHP reaction mixture of the extracts causes it to display dark purple colour coloration.

When the solution (mixture of extraction aliquots and 10 mL of 0.1N HCl) was added with 4 mL ferric chloride, all the aliquots don't show any obvious changes of colour which is the colour from light yellow turn to slightly light brown. Whereas in digestion method with 0.1N concentration of HCl to extract mimosine showed colour conversion into intense violet colour. This shows that there is mimosine present in the solution. However, the soxhlet extraction using ethyl acetate does not show positive result in extracting of mimosine. The hot mantle was set up at high temperature (350°C) and it is observed that ethyl acetate could be easily evaporated as its boiling point is 77.1°C[16]. In other hand, mimosine compound has higher melting point which is 225°C to 228°C[17]. This method does not give expected result due to the need of high temperature for the extraction. Even though this method do not give positive result in extracting mimosine compound, it has been proved that exposing the leaves to high temperature was effective in reducing the direct toxicity of mimosine [18].

3.1.1 pH

It was recorded that the pH value from the extraction by using 0.1N HCl digestion method is 1.83 and those from extraction using soxhlet extraction method is 1.87 and 1.97, using distilled water and ethyl acetate as the solvent respectively. The optimum pH for this experiment should range between 1.5 and 2.5 [19].

In the extraction method, the pH value was measured. The pH value do plays vital role on the intensity of the colour of the solution. In past publication by Matsumoto and Sherman [12], it was stated that if the solution was too acidic which valued lower than 1.5, the colour of the solution was rapidly discharged. However, if the solutions are much more alkaline which exceed more than 2.5 the colour will turn into red and as the pH was increased, the colour will become concentrated until precipitation of ferric hydroxide occurred.

3.2 Quantification of mimosine

3.2.1 Soxhlet extraction with distilled water.

The quantity of mimosine in the extracts was estimated from the calibration curve prepared previously. The equation used to determine the concentration of mimosine as follows;

$$y = 0.0069x + 0.0014 \quad (1)$$

Where the value y is absorbance and x is the concentration.

The highest concentration recorded was by using soxhlet extraction method with distilled water which is 33.66 µg/mL (Table 1). Results tabulated in Table 1 showed differences at confidence level of 95% that there are significant difference between the reading of two extraction for the diluted aliquot of 1-2-3-4 mL labelled as B, C, D and E. However, for 5 mL diluted aliquot labelled F the concentration showed no differences between the two extraction methods.

Table 1. Mean concentration, standard deviation and mean pH of both extraction methods along with results of t-test.

	Soxhlet extraction			HCL digestion			t-test (α=0.05)
	Mean Concentration (µg/mL)	Standard deviation	Mean pH	Mean Concentration (µg/mL)	Standard deviation	Mean pH	
B	7.199	0.865	1.97	4.676	1.126	1.93	H_o rejected
C	15.43	1.997	1.91	9.362	1.671	1.88	H_o rejected
D	23.75	3.882	1.87	14.05	3.808	1.83	H_o rejected
E	30.14	4.699	1.87	16.75	4.870	1.86	H_o rejected
F	33.66	8.204	1.90	20.14	8.303	1.90	H_o accepted

H_o = There is no significant difference between the content of mimosine from two extraction method.
H_A = There is significant difference between the content of mimosine from two extraction method.

This study compares the two extraction methods with differences in time, temperature, mechanism and solvent used. The time taken for extraction in soxhlet extraction and HCl digestion are 8 and 1 hour respectively. The influence of time was not able to be discuss in this study because there are no relevant data recorded. The temperature also could not be analyzed whether it affects the efficiency of mimosine extraction or not but the study conducted in extracting mimosine from *Leucaena leucocephala* showing that degradation of mimosine was possible at 100 °C and higher[20]. This is conflicting with the findings since soxhlet extraction used higher temperature compared to HCl digestion but was able to prove that content was higher with increasing of temperature. The extraction mechanism differ from each other but the only noticeable differences is that soxhlet extraction produced more clarified extract because it separates the samples and solvent. Since HCl digestion mixed both samples and solvent, the extract needs longer time for the precipitation to be settle down inside the storage bottle. Apart from that, soxhlet extraction used distilled water as the solvent instead of HCl and the solvent was proven to be more efficient to extract mimosine compound from the study conducted[21]. The large scale mimosine extraction used distilled water as the solvent and produced 97.5% purity compared to the original method[22] that used HCl as the solvent resulted in 88.75% purity. Various studies was conducted to analyze mimosine by using extraction method of digestion with HCl but the effectiveness of the solvent used often was not discussed.

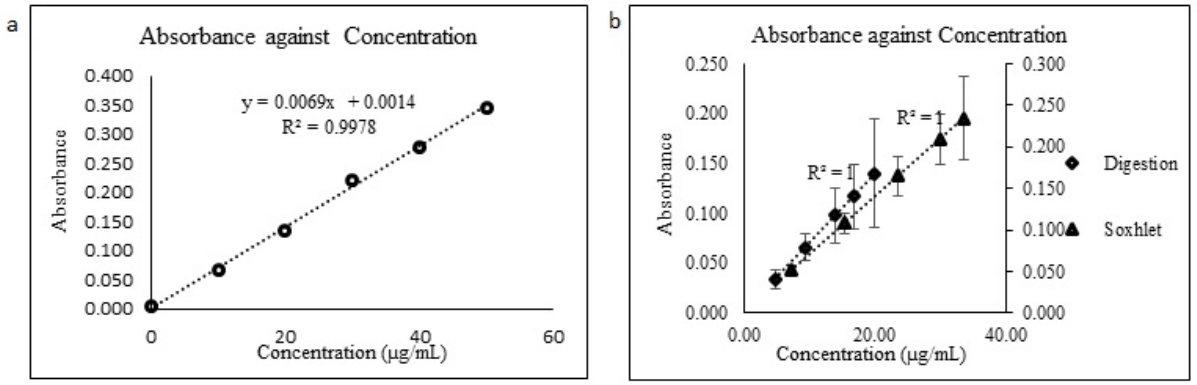

Fig. 1. (a) calibration curve; (b) graph absorbance against concentration of both extraction methods.

Figure 1 (b) showed dissimilarity of the content of mimosine extracted with soxhlet extraction in comparison with HCl digestion. This experiment could not determine the total mimosine content from the leaves of *Leucaena leucocephala* as the aliquots was tested until 5 mL only and there are no zero gradient trend achieved in this experiment. It is also due to the estimation from graph that the quantification method could not be said to be precise enough. However, from soxhlet extraction graph, roughly it can be seen that diluted aliquots gap as it increasing the amount showing less gap between each other. This could indicates that the total concentration of mimosine could be achieved if more aliquot was tested until zero gradient was attained. In contrary, HCl digestion extracts does not showing the same trend as soxhlet digestion extracts. –WHY?

3.2.2 Soxhlet extraction with ethyl acetate.

Since mimosine is a polar compound, this study choose different polar solvents in order to extract mimosine from the leaves which is polarity of distilled water is 10.2 and ethyl acetate is 4.4[23]. Utilization of ethyl acetate as a solvent for soxhlet extraction showed that the highest concentration recorded is 8.47µg/mL.

Ethyl acetate in this experiment showed insignificant result for mimosine extraction, mainly due to unsuitable temperature when using soxhlet extractor. However, by using distilled water which is the most polar solvent, higher concentration of mimosine could be extracted. It is extremely much lower in cost and can extract more rather than any other chemicals. Meanwhile, Siek [24] in his paper suggested chloroform for extraction technique with addition of of alcohol. Thus, in the future it is advisable to use methanol and ethanol as a solvent or co-solvent with water to enhance performance in extraction, safer, self-preservative, evaporated faster and also highly polar.

To compare the extraction efficiency, extraction was also done by using the HCl digestion method. This method was reported to be the fastest [25], safest and no cross-contamination [26] with accurate analytical results [27]. However, from this experiment soxhlet extraction method with distilled water showed positive result and able to extract more mimosine rather than HCl digestion method.

4. Conclusion

As a conclusion, detailed comparison for the variety of time, temperature and mechanism between soxhlet extraction and HCl digestion could only be done through optimization study. Nevertheless, solvent used was the main contributing factor observed in this study for the difference between two extraction methods. Distilled water along with the mechanism of soxhlet extraction was able to extract more mimosine and produced clearer extracts from *Leucaena leucocephala* leaves.

Acknowledgements

The author would like to thank research staffs, Norizan Moez and Mohd Faiz Zakria of Institute of Biological Sciences, for their help and support. University of Malaya Research Grant (RP004B-13BIO) under Sustainability Science Research Cluster is also highly acknowledged.

References

1. Shelton, H. M., & Brewbaker, J. L. (1994). Forage tree legumes in tropical agriculture. In R. C. Gutteridge & H. M. Shelton (Eds.), *Leucaena leucocephala: the Most Widely Used Forage Tree Legume* Wallingford, Oxon: CAB International.
2. Normaniza, O., Faisal, H. A., & Barakbah, S. S. (2008). Engineering properties of Leucaena leucocephala for prevention of slope failure. *Ecological Engineering, 32*(3), 215-221. doi: http://dx.doi.org/10.1016/j.ecoleng.2007.11.004
3. Meena Devi, V.N., Ariharan, V.N., & Nagendra Prasad, P. (2013). Nutritive Value and Potential Uses of Leucaena Leucocephala as Biofuel – A Mini Review. *Research Journal of Pharmaceutical, Biological and Chemical Sciences, 4*(1), 515-521.
4. R. J. Jones, C. G. Blunt and J. H. G. Holmes (1976). Enlarged Thyroid Glands in Cattle Grazing Leucaena Pastures. Tropical Grasslands Vol. 10, No. 2. 113-116
5. Kleipool, R. J. C., & Wibaut, J. P. (1950). Mimosine (leucaenine) 5th communication). *Recueil des Travaux Chimiques des Pays-Bas, 69*(1), 37-44. doi: 10.1002/recl.19500690106
6. Jones, R. J. (1979). The value of Leucaena leucocephala as a feed for ruminants in the tropics. *World Animal Review, 31*, 13-23.
7. Xuan, T. D., Elzaawely, A. A., Deba, F., Fukuta, M., & Tawata, S. (2006). Mimosine in Leucaena as a potent bio-herbicide. *Agron. Sustain. Dev., 26*(2), 89-97.
8. Jones, R. J., & Megarrity, R. J. (1986). Successful transfer of DHP-degrading bacteria from Hawaiian goats to Australian ruminants to overcome the toxicity of leucaena. *Australian Veterinary Journal, 63*, 259-262.
9. Hegarty, M. P., Lee C. P., Christie, G. S., Court, R. D., & Haydock, K. P. (1979). The goitrogen 3-hydroxy4(1H)pyridone, a ruminal metabolite of leucaena: effects in rats and mice. *Australian Journal of Biological Sciences, 32*(27-40).
10. Harinder, P.S., Makkar, P., & Siddhuraju, K. B. (2007). L-Mimosine (b-(3-Hydroxy-4-Pyridone-1-yl)-L-Alanine *Methods in Molecular Biology; Plant Secondary Metabolites*. Totowa, New Jersy: Humana Press.

11. Von, U., ter Meulen, & El Harith, E. A. (1985). Mimosine - a factor limiting the use of Leucaena leucocephala as an animal feed.
12. Matsumoto, H., & Sherman, G. D. (1951). A rapid colorimetric method for the determination of mimosine. *Archives of Biochemistry and Biophysics, 33*(2), 195-200. doi: http://dx.doi.org/10.1016/0003-9861(51)90098-7
13. Palmer, B., Jones, R., Poathong, S., & Chobtang, J. (2010). Within—country variation in the ability of ruminants to degrade DHP following the ingestion of Leucaena leucocephala—a Thailand experience. *Tropical Animal Health and Production, 42*(2), 161-164. doi: 10.1007/s11250-009-9398-4
14. Kostermans, D. (1947). The structure of mimosine. *Recueil des Travaux Chimiques des Pays-Bas, 66*(2), 93-98. doi: 10.1002/recl.19470660206
15. Allison, M. J., Hammond, A. C., & Jones, R. J. (1990). Detection of ruminal bacteria that degrade toxic dihydroxypyridine compounds produced from mimosine. *Applied and Environmental Microbiology, 56*(3), 590-594.
16. PubChem. (2004a). Ethyl acetate. Retrieved 29 May, 2015, from http://pubchem.ncbi.nlm.nih.gov/compound/ethyl_acetate#section=Top
17. PubChem. (2004b). L-mimosine. Retrieved 29 May, 2015, from http://pubchem.ncbi.nlm.nih.gov/compound/440473#section=Top
18. Wee, K. L., & Wang, S. (1987). Effect of post-harvest treatment on the degradation of mimosine in Leucaena leucocephala leaves. *Journal of the Science of Food and Agriculture, 39*(3), 195-201.
19. Makkar, H. P., Siddhuraju, P., & Becker, K. (2007). *Trypsin Inhibitor*: Springer.
20. Wills, R. B. H., & Tangendjaja, B. (1981). Effect of pH and Temperature on the Degradation of Mimosine and 3-Hydroxy-4(1H)-Pyridone. *Phytochemistry, 20*, 2017-2018.
21. El Harith, E. A., Szyszka, M., Günther, K. D., & ter Meulen, U. (1987). A Method for Large Scale Extraction of Mimosine1,2. *Journal of Animal Physiology and Animal Nutrition, 57*(1-5), 105-110. doi: 10.1111/j.1439-0396.1987.tb00014.x
22. Yoshida, & Ruth, K. (1944). *A Chemical and Physiological Study on the Nature and Properties of the Toxic Principle in Leucaena glauca (Koa Haole)*. University of Minnesota.
23. Paul Sadek The HPLC Solvent Guide Wiley-Interscience, 2002.
24. Siek, T. J. (1978). Effective use of organic solvents to remove drugs from biologic specimens. *Clinical toxicology, 13*(2), 205-230.
25. Hedren, E., Diaz, V., & Svanberg, U. (2002). Original Communications-Estimation of carotenoid accessibility from carrots determined by an in vitro digestion method. *European journal of clinical nutrition, 56*(5), 425-430.
26. Parr, J. F., Dolic, V., Lancaster, G., & Boyd, W. E. (2001). A microwave digestion method for the extraction of phytoliths from herbarium specimens. *Review of Palaeobotany and Palynology, 116*(3), 203-212.
27. Melaku, S., Dams, R., & Moens, L. (2005). Determination of trace elements in agricultural soil samples by inductively coupled plasma-mass spectrometry: microwave acid digestion versus aqua regia extraction. *Analytica Chimica Acta, 543*(1), 117-123.