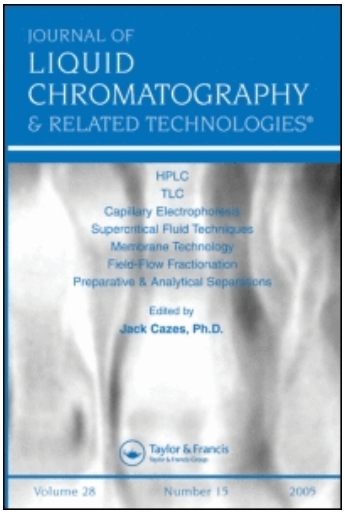

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Quantitative HPLC Methods for Gallic Acids of *Phyllanthus* (Euphorbiaceae)

Chao-Ying Lee[a]; Tai-Hui Chiu[b]; Shih-Wei Tsai[c]
[a] Graduate Institute of Chinese Pharmaceutical Sciences and School of Pharmacy, College of Pharmacy, China Medical University, Taichung, Taiwan, R.O.C [b] School of Pharmacy, College of Pharmacy, China Medical University, Taichung, Taiwan, R.O.C [c] Graduate Institute of Pharmaceutical Chemistry, College of Pharmacy, China Medical University, Taichung, Taiwan, R.O.C

To cite this Article Lee, Chao-Ying , Chiu, Tai-Hui and Tsai, Shih-Wei(2005) 'Quantitative HPLC Methods for Gallic Acids of *Phyllanthus* (Euphorbiaceae)', Journal of Liquid Chromatography & Related Technologies, 28: 18, 2965 — 2977
To link to this Article: DOI: 10.1080/10826070500274604
URL: http://dx.doi.org/10.1080/10826070500274604

Journal of Liquid Chromatography & Related Technologies®, 28: 2965–2977, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500274604

Quantitative HPLC Methods for Gallic Acids of *Phyllanthus* (Euphorbiaceae)

Chao-Ying Lee
Graduate Institute of Chinese Pharmaceutical Sciences and School of Pharmacy, College of Pharmacy, China Medical University, Taichung, Taiwan, R.O.C

Tai-Hui Chiu
School of Pharmacy, College of Pharmacy, China Medical University, Taichung, Taiwan, R.O.C

Shih-Wei Tsai
Graduate Institute of Pharmaceutical Chemistry, College of Pharmacy, China Medical University, Taichung, Taiwan, R.O.C

Abstract: There are many different shrubs and herbaceous species of *Phyllanthus* (Euphorbiaceae) in Taiwan.

We set up a quantitative method to determine the quantity of gallic acid (GA). This method has a high degree of sensitivity, reproducibility, precision, and recovery rate. In order to ensure the quality of the *Phyllanthus*, we can apply this method to evaluate the quality of these plants.

We sampled several different *Phyllanthus* plants in Taiwan, including *P. myrtifolius, P. multiflorus, P. amarus, P. debilis, P. embergeri, P. tenellus, and P. urinaria* subsp. *urinaria*, and measured one of the major active components—GA via the high performance liquid chromatography (HPLC) method. The result shows a great range of GA contained in these samples, although the quantity of GA varies with the place and season of sample origin. The samples collected in southern Taiwan during summer contained more GA than samples from other places and seasons. For example, the weight of GA in *P. debilis* from Pingtung was 88.13449 μg (0.018%) and the weight of GA in *P. embergeri* from Taipei was 2002.991 μg (0.401%). The difference between the weights of GA in two species can be as much as 20 times.

Keywords: *Phyllanthus*, Gallic acid, HPLC, Content determination

Address correspondence to Chao-Ying Lee, Graduate Institute of Chinese Pharmaceutical Sciences, College of Pharmacy, China Medical University, Taichung, Taiwan 404, R.O.C. E-mail: cylee@mail.cmu.edu.tw

INTRODUCTION

The *Phyllanthus*, a genus with a great number of species in Euphorbiaceae; it contains over 650 species[1] and is widely distributed in tropical, subtropical, and temperate areas.

There are fourteen species[1] of *Phyllanthus* reported in Taiwan, including *P. acidus, P. emblica, P. myrtifolius, P. oligospermus, P. multiflorus (P. reticulatus*), *P. amarus* (*P. niruri*), *P. debilis, P. embergeri, P. hookeri, P. tenellus, P. urinaria* subsp. *nudicarpus, P. urinaria* subsp. *urinaria, P. ussuriensis* (*P. matsumurae*), and *P. virgatus* (*P. simplex*). Their similarity in morphology confuses people who try to tell different species apart. The *Phyllanthus* has little side effects and toxicity, and their common name in Taiwan is "Chu-Zi-Tsao, Jih-Kai-Yeh-Pi, Chen-Chu-Tsao."[2]

The *Phyllanthus* has been used all over the world for a long time, (i.e., for the treatment of jaundice, hepatitis in India). They have the heat-clearing and damp-drying action (*P. virgatus*)[3] inducing diuresis, excreting dampness to arrest diarrhea, clearing away toxins and subduing swelling, improving the acuity of vision (*P. urinaria*) and being able to remedy urinary tract infection (*P. hookeri*).[3]

Venkateswaran[4] has proposed that an aqueous extract of *P. niruri* can inhibit hepatitis B and woodchuck hepatitis viruses. The rate of recovery of a woodchuck from WHsAg (woodchuck hepatitis surface antigen) with an aqueous extract of *P. niruri* is perfect (100%), with no rebound phenomenon observed. *P. amarus* plants were prepared and tested as previously described by S.P. Thyagarajan[5] on HBsAg (hepatitis B surface antigen) patients. The rate of recovery on 27 HBsAg patients who took the powder of *P. niruri* is 66%. But, not all the results of these papers were satisfying. Amorn Leelarasamee[6] presented results about a 30% HBeAg decrease, but not complete disappearance. This result failed to meet the significant standard in pathology.

From the document mentioned above, we confirmed gallic acid (GA) is one of the active components in *Phyllanthus* plants. GA is the most commonly used component, which has pharmacological properties as an antimicrobial, astringent and obstruent agent. In this paper, we use high performance liquid chromatography (HPLC) to determine the quantity of GA contents in *Phyllanthus* plants. This experimental result provided us with a new indicator and a controlled method to examine the quality of *Phyllanthus* plants.

EXPERIMENTAL

Plant Materials

Phyllanthus plants collected in Taiwan, included *P. myrtifolius, P. multiflorus, P. amarus, P. debilis, P. embergeri, P. tenellus, P. urinaria* subsp. *urinaria*,

etc. The voucher specimens were deposited in the Herbarium of National Museum of Natural Science (TNM), Taichung (Table 1).

The fresh material was first dried at room temperature, and then dried in an oven untill it reached constant weight. The GA contents were calculated as a percentage of the dry weights.

Reagents

GA was obtained from Ricedel-de Haën Ag Seelze-Hannover, methanol and phosphoric acid were purchased from Merck, water (Milli Q system) and internal standards (I.S., Acetaminophen) were used. Other reagents or solvents used were analytical reagent grade or HPLC grade.

High Performance Liquid Chromatography[7,8]

The instruments used in this research were a Jasco 851-AS intelligent sampler, Jasco PU-980 intelligent HPLC pump*2, equipped with Jasco UV-975 intelligent UV/Vis (280 nm) detector. Analyses were performed with an 250 mm × 4 mm I.D. column packed with Rp-18 (Merck LiChroCART 250-4). Samples were filtered through a 0.22 μm Minipore filter, than 20 μL was injected into a column. The chart speed was 10 mm/min, mobile phase MeOH : 0.1%H_3PO_4 = 3 : 97, flow rate 1 mL/min.

GA Extraction Method

The powder of *Phyllanthus*, 0.5 g, with 50 mL H_2O, was extracted 3 times repeatedly. Then 3 solutions were combined into 150 mL, and filtered with a 0.22 μM Minipore Filter and 20 μL sample injected into the HPLC.

Table 1. Collection of *Phyllanthus* plants

Species	Collector
P. myrtifolius	TNM C.Y. Lee129
P. multiflorus	TNM C.Y. Lee169
P. amarus	TNM C.Y. Lee131
P. debilis	TNM C.Y. Lee 26
P. embergeri	TNM C.Y. Lee132
P. tenellus	TNM C.Y. Lee133
P. urinaria subsp. *urinaria*	TNM C.Y. Lee138

Accuracy and Precision Test

In search of precision, we used the mobile phase (MeOH : 0.1%H_3PO_4 = 3 : 97) to quantify GA. Three solutions with different concentrations were injected four successive times in the same day (intraday) and three successive times on different days of the week (interday). Each solution was injected 12 times in total.

The data are shown in Tables 2 and 3.

Recovery Tests

We prepared the water solution of GA at the concentration 8.8 μg/mL, and named it solution A. Then we dissolved 0.5 g *P. debilis* powder into 50 mL of water, and mixed it with 50 mL of solution A. After heating the mixture for 15 minutes, the sample solution was ready. Then, we repeated this procedure 3 times to gather 3 sample solutions as *P. debilis* 1, 2, and 3 (shown in Table 4). We prepared our blank solution by dissolving 0.5 g *P. debilis* in 50 mL water and heated it for 15 minutes. Then, we took 0.9 mL of solution from each of the 3 samples and blank solutions, and mixed them with 0.1 mL I.S. after filtering all the mixtures and sampling them.

Stability Study

We prepared the water solution of GA at the concentration of 3.8 μg/mL. A definite volume of I.S. solution was added to an aliquot of GA by means of a lambda pipette and thoroughly mixed. The mixed solution was then chromatographed once an hour for twelve hours consecutively.

Table 2. Intra-day analyses of GA

GA conc. (μg/mL)	Day	n	Average ± S.D.	RSD. (%)
1.16	1	4	0.089577 ± 0.000751	0.84
	2	4	0.085734 ± 0.000338	0.39
	3	4	0.085311 ± 0.003036	3.56
5.8	1	4	0.381981 ± 0.002163	0.57
	2	4	0.376684 ± 0.001238	0.33
	3	4	0.384884 ± 0.01028	2.67
11.6	1	4	0.779924 ± 0.001491	0.19
	2	4	0.77412 ± 0.001758	0.23
	3	4	0.77547 ± 0.004098	0.53

n: Numbers of injection in one day.

Table 3. Inter-day analyses of GA

GA conc. (μg/mL)	Day	n	Average ± S.D.	RSD (%)
1.16	7	12	0.086874 ± 0.003515	4.05
5.8	7	12	0.381183 ± 0.006978	1.83
11.6	7	12	0.776505 ± 0.003641	0.47

n: Numbers of injection in one week.

Calibration Curve Setup

We prepared six separate GA solutions. The concentration of these solutions were 0.58, 2.32, 3.48, 4.64, 5.8, and 11.6 μg/mL; and the I.S., acetaminophen was 45 μg/mL. A definite volume of I.S. solution was added to an aliquot of GA by means of a lambda pipette into 1 mL volumetric flasks, and thoroughly mixed individually. After chromatography of the mixed solutions, we obtained the integrated peak areas of GA and I.S (Figure 1).

Calculation of GA in Samples

We extracted 0.5 g plant powder with 50 mL water, then mixed the plant extract with I.S. (45 μg/mL), and performed chromatography on the solution (Figure 2).

The amount of GA in this sample was calculated in 3 steps.

1. We calculated the GA to I.S. peak area ratio from the chromatogram.
2. Then we obtained the corresponding peak area ratio of GA to I.S. from the calibration curve.
3. After we multiplied the corresponding peak area ratio to the concentration of GA in sample, we obtained the weight of GA in the sample.

The obtained value was then converted to weight % of GA in dry samples.

Table 4. Recovery tests

	GA/I.S.1	GA/I.S.2	GA/I.S.3	Average	Recovery (%)
Blank	0.226855	0.228683	0.221304	0.225614	
P. debilis 1	0.736287	0.739911	0.736935	0.737711	100.50
P. debilis 2	0.719791	0.718682	0.72513	0.721201	97.26
P. debilis 3	0.748364	0.743445	0.762569	0.751459	103.19
RSD					2.41

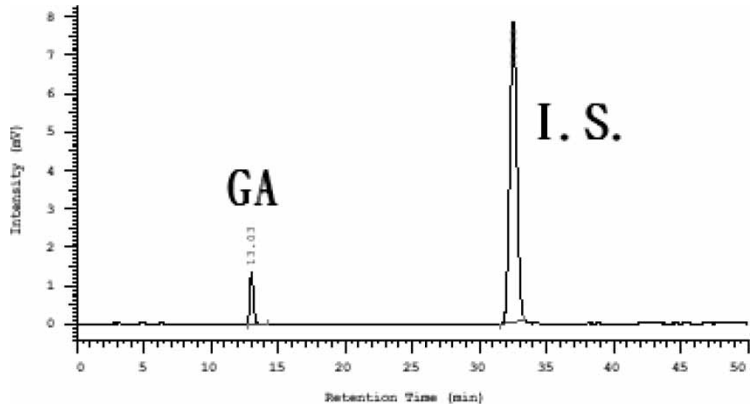

Figure 1. Typical HPLC of GA and acetaminophen 250 mm × 4 mm I.D. column packed with Rp-18, mobile phase MeOH : 0.1%H_3PO_4 = 3 : 97, 1 mL/min.

Limit of Detection

We diluted GA into 10.4, 4.16, 3.12, 2.08, 0.728, 0.624, 0.52, 0.416, 0.312 μg/mL, then injected each 20 μL solution 3 times. The GA peak area's RSD was calculated to determine the lowest concentration that can be detected, the RSD >5% not being a reliable data.

RESULTS

HPLC Method Validation

Accuracy and Precision

We calculated the relative standard deviation (RSD) by taking the ratio of GA absorption area and I.S. (Acetaminophen) absorption area individually.

The reproducibility of this quantitative result is excellent. The area ratio relative standard deviation range of intraday and interday was acceptable (Tables 2 and 3).

The data showed good accuracy and precision in this HPLC method when quantifying GA.

Recovery Tests

We obtained the results shown in Table 4.

We obtained the concentration of GA in samples and compared these values with the concentration of solution we added to calculate the recovery rate. The peak area ratio of GA to I.S. was 0.509571. From the results we

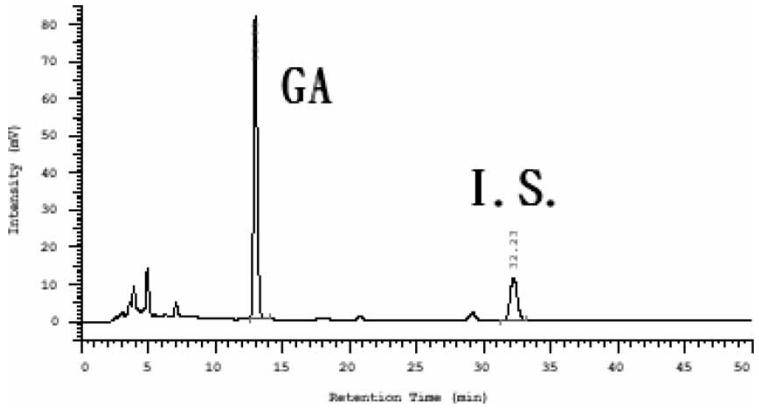

Figure 2. Typical HPLC of *Phyllanthus* extracts 250 mm × 4 mm I.D. column packed with Rp-18, mobile phase MeOH : 0.1%H_3PO_4 = 3 : 97, 1 mL/min.

could see an excellent recovery rate between 97.26% ~ 103.19%; and a low standard deviation at 2.41%. The recovery was very successful (Table 4).

Stability Study

From Figure 3 we can see that the amount of GA remains stable for 12 hrs. Therefore, samples should be treated within 12 hrs in order to examine the GA content precisely.

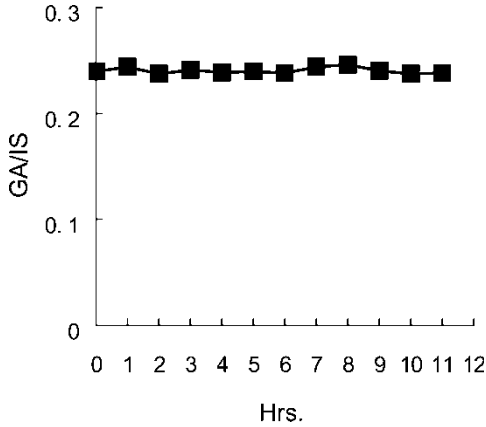

Figure 3. Stability of GA in 12 hrs.

Calibration Curves

The calibration curve was based on the ratio of peak areas of GA to I.S. (x) versus the concentration of the GA (Y) (Figure 4). The calibration relationship is $y = 15.436X + 0.0692$, $R^2 = 0.9998$.

The linear range of gallic acid was $0.58 \sim 11.6\,\mu g/mL$.

Limit of Detection

From the data shown in Table 5, it could be inferred that the concentration below $0.416\,\mu g/mL$ is not detectable.

Quantification of the GA in *Phyllanthus* Plants

The data of GA contents of *Phyllanthus* in Taiwan are listed in Table 6.

DISCUSSION

Extraction Method Selection

GA is a highly water soluble compound. Most of the reported cases which had high therapeutic effects[4,5,7–9] were using the water extracted *P. debilis.* Therefore, we used water as the solvent to extract *P. debilis* after trying three different methods to extract water soluble contents, including:

1. Extraction with ultrasonics for 30 mins.[10]
2. Stirring for 18 hours at room temperature.[11]
3. Heating for 15 mins.[12–14]

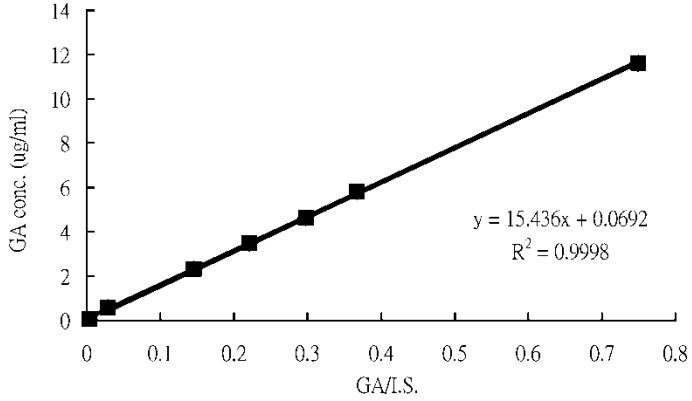

Figure 4. Calibration curve.

Table 5. RSD of GA peak area

Concentration (μg/mL)	RSD (%)
103	4.346137
82.4	0.239699
61.8	0.048597
41.2	0.620424
20.6	0.84483
10.3	3.312308
8.24	37.51528
6.18	33.3946
4.12	undetectable

Due to its effectiveness and the short time required, we chose the heating method in this experiment.

In order to make sure we extracted the entire GA from the sample, we extracted the powder of *P. debilis* (0.5 g with 50 mL H_2O), repeatedly, 4 times. Each extraction was injected 3 times into the HPLC. From Table 7, we can see from all 3 samples, that we could not detect any GA from the

Table 6. Contents of GA in *Phyllanthus* plants

Samples	Date	Place	Weight (μg)	Contents (%)
P. multiflorus	2002/11/18	Pingtung	650.184	0.130
	2002/12/22	Pingtung	638.588	0.128
P. myrtifolius	2003/1/27	Taipei	550.524	0.110
	2003/1/30	Tainan	1010.028	0.202
P. amarus	2001/8/12	Tainan	503.507	0.101
	2002/11/4	Nantou	489.984	0.098
	2002/11/10	Taichung	329.497	0.066
	2002/11/16	Pingtung	117.627	0.024
	2003/2/1	Tainan	339.492	0.068
P. debilis	2002/8/22	Taichung	503.929	0.101
	2002/8/28	Taipei	561.833	0.112
	2002/11/16	Pingtung	88.134	0.018
	2002/11/25	Taichung	360.548	0.072
P. embergeri	2002/8/28	Taipei	2002.991	0.4101
P. tenellus	2002/5/11	Taichung	924.744	0.185
	2002/11/11	Taichung	333.408	0.067
	2002/11/26	Taichung	378.435	0.076
P. urinaria ssp. urinaria	2002/8/15	Taichung	393.281	0.079
	2002/11/5	Taichung	257.970	0.052

Table 7. Limit of extraction

GA/I.S.	1st	2nd	3rd	4th	Total
Sample 1	79.35022	18.09709	2.552684	0	100
Sample 2	79.9471	16.57529	3.477611	0	100
Sample 3	83.52415	12.46541	4.010441	0	100
RSD	2.27711	15.13866	17.99449	0	0

fourth HPLC result. After observing this experiment, we confirmed that GA was completely extracted with 50 mL H_2O within three times.

Comparison of GA Contents in the Samples Collected in Different Places at the Same Time, its Contents Were Determined by Methods III and IV

P. myrtifolius samples (Table 8) were collected from Tainan and Taipei in January. The GA content in the samples from Taipei was only one-half of the GA in the samples from Tainan.

P. amarus samples (Table 8) were collected from Taichung, Nantou, and Pingtung in November. The GA quantity in the samples from Pingtung was very low, merely one-third of the quantity in the samples from Taichung. The GA content ratio of *P. amarus* in Taichung over Nantou is around 3 : 5.

P. debilis samples (Table 8) were collected from Pingtung and Taichung in November. The GA content in the samples from Pingtung is only one-fourth of those in the samples from Taichung, but the GA content in the samples from Taichung were identical to the content in *P. debilis* samples from Taipei collected in August.

Table 8. Contents of GA in *Phyllanthus* plants

Samples	Date	Place	Weight (μg)	Contents (%)
P. myrtifolius	2003/1/27	Taipei	550.524	0.110
	2003/1/30	Tainan	1010.028	0.202
P. amarus	2002/11/16	Pingtung	117.627	0.024
	2002/11/10	Taichung	329.497	0.066
	2002/11/4	Nantou	489.984	0.098
P. debilis	2002/11/16	Pingtung	88.134	0.018
	2002/11/25	Taichung	360.548	0.072
	2002/8/22	Taichung	503.929	0.101
	2002/8/28	Taipei	561.833	0.112

Table 9. Contents of GA in *Phyllanthus* plants

Samples	Date	Place	Weight (μg)	Contents (%)
P. multiflorus	2002/12/22	Pingtung	638.588	0.128
	2002/11/18	Pingtung	650.184	0.130
P. amarus	2003/2/1	Tainan	339.492	0.068
	2001/8/12	Tainan	503.507	0.101
P. debilis	2002/11/25	Taichung	360.548	0.072
	2002/8/22	Taichung	503.929	0.101
P. tenellus	2002/11/11	Taichung	333.408	0.067
	2002/11/26	Taichung	378.436	0.076
	2002/5/11	Taichung	924.744	0.185
P. urinaria ssp. urinaria	2002/11/5	Taichung	257.970	0.052
	2002/8/15	Taichung	393.281	0.079

Comparison of GA Contents in the Samples Collected at Different Times in the Same Place, its Contents were Determined by Methods III and IV

The samples of *P. multiflorus* in Table 9 were collected in November and December from Kenting. The ratio of GA in these samples is about the same.

The *P. amarus* samples in Table 9 were collected from Tainan at different times (February and August). The GA contents of the collected samples in August were higher than the samples in February. The ratio of GA contents in samples colleted in August/February was around 5 : 3.

The *P. debilis* samples in Table 9 were collected from Taichung, at different times (August and November). The GA content of the samples in August was higher than it was in November, and the ratio of August/November GA content was about 5 : 3.5.

The samples of *P. tenellus* in Table 9 were collected in November and May from Taichung, and a big difference between the ratio of GA in these samples was observed. The GA ratio in sample collected in May was three times higher than it was in sample collected in November (1 : 3).

Table 10. Contents of GA in *Phyllanthus* plants

Samples	Date	Place	Weight (μg)	Contents (%)
P. debilis	2002/11/16	Pingtung	88.134	0.018
P. embergeri	2002/08/28	Taipei	2002.991	0.4101

The samples of *P. urinaria* subsp. *urinaria* in Table 9 were collected in August and November from Taichung. The ratio of the GA contents ratio in samples collected in August/November was about 4:2.5.

Comparison of GA Contents in *Phyllanthus* Genus

After applying HPLC to all samples we collected for further investigation of GA content, we achieved an integral area of GA.

Among *Phyllanthus* genus plants in Taiwan, the contents of GA were very different. For example, in the powder of 0.5 g *Phyllanthus* plants (Table 10), the weight of GA in *P. debilis* from Pingtung was 88.13449 μg (0.018%) and the weight of GA in *P. embergeri* from Taipei was 2002.991 μg (0.401%). The difference between the weights of GA in two species can be as much as 20 times.

CONCLUSIONS

Comparison of GA Contents in the Samples Collected at Different Environments

The GA contents of *Phyllanthus* plants from southern Taiwan were higher than in the same species from northern Taiwan in the mountainous areas (Table 8). However, it's a different story at the seaside; the GA content of *Phyllanthus* plants during summer is less than in the other seasons. The potential reason is that GA turns into a polymer structure under the humidity at the seaside.

The summer in Taiwan is from May to August. During this time the plants grow faster and have a higher metabolism rate. Owing to latitudes, the temperature in southern Taiwan is higher than in northern Taiwan. Therefore, we could infer that GA contents in *Phyllanthus* plant would be affected by the temperature of the environment in which it grows (Table 8).

Comparison of GA Contents in *Phyllanthus* Genus

Among *Phyllanthus* genus plants in Taiwan, the contents of GA were very different. The difference between the weights of GA in two species can be as much as 20 times (Table 10).

Combining these two consequences could give us a new way to evaluate herbs for their medicinal potency.

ACKNOWLEDGMENT

This work was supported by the Grant CMU93-P-12 of China Medical University, Taiwan, R.O.C.

REFERENCES

1. Tsai, S.W. *The Taxonomic Study of Phyllanthus (Euphorbiaceae) in Taiwan*; Masters Thesis, 2003.
2. Chiu, N.Y.; Chang, K.H. *The Illustrated Medicinal Plants of Taiwan*, 1st Ed.; SMC Publishing Inc: Taipei, 1983; Vol. 1–6.
3. *Yunnan Medicine Resource Blue Book*, 1st Ed.; Yunnan Traditional Medicine Co Beijing. Science Press, 1993; 286–288.
4. Venkateswaran, P.S.; Millman, I.; Blumberg, B.S. Proc. Natl. Acad. Sci. U.S.A. **1987**, *84*, 274.
5. Thyagarajan, S.P.; Subramanian, S.; Thirunalasundari, T.; Venkateswaran, P.S. Lancet **1988**, 764.
6. Leelarasamee, A.; Trakkulsomboon, S.; Maunwongyathi, P.; Somanabandhu, A.; Pidetcha, P.; Matrakool, B.; Lebnak, T.; Ridthimat, W.; Chandanayingyong, D. Lancet. **1990**, *335*, 1600.
7. Huang, Z.C.; Deng, X.L.; Zhu, Y.T.; Zhang, M.Y.; Liu, N.; Guo, X.B. J. of Guangzhou Univ, Tradit. Chinese Med. **2000**, *17*, 260.
8. Zhou, S.W.; Xu, C.F.; Zhou, N.; Huang, Y.P.; Huang, L.Q.; Chen, X.H.; Hu, Y.M.; Liao, Y.Q. China J. of Chinese Materia Medica (Zhongguo Zhongyao Zazhi) **1997**, *22*, 109.
9. Blumberg, B.S.; Millman, I.; Venkateswaran, P.S.; Thyagarajan, S.P. Vaccine **1990**, *8*, s86.
10. Zhou, P. Lishizhen Medicine and Materia Medica Research. **2001**, *12*, 291.
11. Zhang, L.Z.; Wang, X.Q.; Jia, H.; MA, J.Z. J. Beijing Univ. of TCM. **2000**, *23*, 46.
12. Zuo, Y.G.; Chen, H.; Deng, Y.W. Talanta. **2002**, *57*, 307.
13. Wang, J.C.; Fang, K.H.; Pan, J.H.; Luo, L. J. Nanjing Univ. of TCM. **1998**, *14*, 224.
14. Zhao, S.P.; Fu, G.X. J. China-Japan Friendship Hospital **1998**, *12*, 3.

Received January 20, 2005
Accepted May 19, 2005
Manuscript 6571