CHAPTER V

DISCUSSIONS

Total isoflavonoid contents including puerarin, daidzin, genistin, daidzein and genistein of 5-clone cultivated *P. mirifica* collected in three seasons during the year of 2005; summer in April, rainy season in August and winter in December has been investigated in this study. There were variations of weather, with the highest average temperature in April (31.3 °C) and lowest average temperature in December (25.1 °C). The most differentiation of maximum and minimum temperature of 11.5 °C was found in April that could initiate heat shock of gene and influent on isoflavonoids synthesis and storage during summer. The maximum rainfall amount of 114.8 mm was detected in August and the minimum one was found in April (0.9 mm). This event could initiate water stress on plants and its chemical contents during summer especially in April.

The 5 major isoflavonoid contents were analyzed by HPLC and the results indicated that there were seasonal variations of individual and total isoflavonoids contents in the same plant clone. For example, in *P. mirifica* collected in summer, the maximum amount of puerarin and total isoflavonoid contents were found in PM-IV (127.22±16.47 and 181.26±22.24 mg/100 g, respectively), daidzin and genistin in PM-II (54.22±26.46 and 18.97±11.97 mg/100 g, respectively); and daidzein and ginistein in PM-III (19.36±5.35 and 5.61±1.79 mg/100 g, respectively). Amomg *P. mirifica* collected in rainy season, the highest content of puerarin and total isoflavonoid were found in PM-III (132.05±13.79 and 191.16±10.21 mg/100 g, respectively), daidzin in PM-V (38.01±2.26 mg/100 g), genistin and daidzein in PM-II (26.40±9.23 and 16.70±4.70 mg/100 g, respectively); and genistein in PM-I (15.23±3.66 mg/100 g). Meanwhile, in *P. mirifica* collected in winter, the maximum amount of puerarin, daidzin, genistin and total isoflavonoid contents exhibited in PM-IV (168.69±48.13, 50.09±11.13, 16.61±1.35 and 249.31±60.63 mg/100 g, respectively), daidzein in PM-I (14.58±1.46 mg/100 g), and genistein in PM-V (2.61±1.77 mg/100 g).

Among three seasons, the highest average amount of puerarin, daidzin and total isoflavonoid contents were found in *P. mirifica* collected in winter; whereas, the highest average amount of ginistin and genistein were detected in *P. mirifica* collected in rainy season. But the most content of daidzein from *P. mirifica* collected in summer was detected. Puerarin was the most abundant glycoside isoflavonoid in the tubers, thus this

chemical could represent the accumulation trend of total isoflavonoid. High temperature and lack of water from rainfall could affect the accumulation of isoflavonoid contents in tubers.

Genistin and genistein was the highest amount found in PM-I collected in rainy season. Whereas, puerarin in PM-III collected in rainy and winter seasons was higher than those from the ones harvested in summer. Also, genistin in PM-IV collected rainy and winter seasons was higher than those from the ones collected in summer time. Futhermore, daidzin and genistin in PM-V collected in rainy season and winter was higher than the ones harvested in summer. The highest amount of daidzein and total isoflavonoid was found in PM-V collected in winter. No significant difference of isoflavonoid contents in PM-II collected in all seasons was observed.

Also, the results indicated that there were the significant differences of the mean value of isoflavonoid contents in different clones of *P. mirifica*. The highest level of puerarin and total isoflavonoid contents were found in PM-IV, genistin in PM-II, daidzein in PM-I, PM-II and PM-III; and genistein in PM-I. However, there was no significant difference of daidzin content among five clones of *P. mirifica*.

Aglycoside, glycoside, the ratio of aglycoside/aglycoside, the ratio of glycoside/aglycoside, the ratio of aglycoside and glycoside/puerarin from *P. mirifica* were analyzed. *P. mirifica* collected in summer, the maximum of aglycoside and the ratio of aglycoside/glycoside was found in PM-I (15.35±1.71 and 0.36±0.05, respectively), glycoside and the ratio of glycoside/aglycoside in PM-IV (66.70±12.41 and 5.43±1.40, respectively), and the ratio of aglycoside and glycoside/puerarin in PM-I and PM-III (0.77±0.28, 0.77±0.17, respectively). But, *P. mirifica* collected in rainy season, the maximum of aglycoside and the ratio of aglycoside/glycoside was found in PM-III (24.97±3.56 and 0.64±0.08, respectively), glycoside in PM-II (73.19±38.42), the ratio of glycoside/aglycoside in PM-IV (7.40±0.07), and the ratio of aglycoside and glycoside/puerarin in PM-I (1.00±0.23). Meanwhile, *P. mirifica* collected in the winter season, the highest of aglycoside and the ratio of aglycoside/glycoside were found in PM-I (29.36±6.51 and 0.62±0.21), glycoside and the ratio of aglycoside and glycoside/puerarin in PM-I (58.48±18.10 and 0.99±0.14, respectively), and the ratio of glycoside/aglycoside in PM-IV (4.55±0.97).

The significant difference of the mean values of aglycoside, glycoside, the ratio of aglycoside/aglycoside, the ratio of glycoside/aglycoside, the ratio of aglycoside and

glycoside/puerarin in *P. mirifica* harvested from three seasons were detected. The maximum figure of aglycoside was exhibited in *P. mirifica* collected in rainy season but the most abundance ratios of aglycoside/glycoside and of aglycoside and glycoside/puerarin were detected *P. mirifica* harvested in summer time. No significant difference of glycoside and the ratio of glycoside/aglycoside were found in *P. mirifica* that collected from different three seasons.

Obviously, PM-III collected in summer was found to contain the highest amount of aglycoside, the ratio of aglycoside/glycoside and the ratio of aglycoside and glycoside/puerarin. But the highest amount of the ratio of glycoside/aglycoside was found in PM-III collected in winter. The highest amount of the ratio of aglycoside/glycoside was found in PM-V harvested in summer, but their glycoside detected from PM-V collected in rainy and winter seasons was higher than those from the ones harvested in summer.

There was significant difference of the mean value of aglycoside, glycoside, the ratio of aglycoside/glycoside, the ratio of glycoside/aglycoside, the ratio of aglycoside and glycoside/puerarin in five clones of *P. mirifica*. The aglycoside and ratio of aglycoside and glycoside/puerarin in PM-I, PM-II and PM-III were higher than those from the other clones. The highest amount of the ratio of aglycoside/glycoside was found in PM-I whereas the highest amount of the ratio of glycoside/aglycoside was detected in PM-V. Interestingly, no significant difference of glycoside amount in *P. mirifica* among five clones was detected.

Isoflavonoid and total isoflavonoid contents in *P. mirifica* were not correlated with the change of temperature (C°) but the influence of rainfall amount during the studied period in Ratchaburi province played a great role with isoflavonoid contents storage in tubers. There were correlations between genistin and the ratio of glycoside and aglycoside with the amount of rainfall and negative correlation between ratio of aglycoside/glycoside with the amount of rainfall in *P. mirifica* collected in rainy season. The correlation of aglycoside and glycoside against puerarin were found with the amount of rainfall in *P. mirifica* collected in winter. But no correlation between isoflavonoids and weather in *P. mirifica* collected summer was observed.

The antioxidant activity test of the 5 clones of plant extracts by DPPH assay revealed that the plant exhibited antioxidant activity in different degree. The plant extracts showed significant lower antioxidant activity than α–tocopherol. The

isoflavonoids were relatively poor hydrogen donors compared with the other estrogenic compounds. The data revealed that plant extract had low scavenging activity and IC50 > 1000 μg/ml. At concentration 6000 μg/ml, PM-I collected in summer and winter, PM-II collected in rainy season showed the highest percentage of scavenging. There were no significant differences of the mean value of scavenging activity among *P. mirifica* collected in theree seasons.

No significant difference of the value of scavenging activity among five clones of *P. mirifica* in every concentration was tested. PM-I and PM-IV had the higher scavenging activity than others clones at 375 μg/ml. While PM-I collected in summer at concentration 750, 1500, 3000 and 6000 μg/ml was the highest.

There was correlation between the percentages of scavenging at the concentration 750 μg/ml of *P. mirifica* collected in summer with the amount of aglycoside content. There was no correlation between the percentage of scavenging and isoflavonoid contents in *P. mirifica* collected in rainy season and winter.

There were at least 3 approaches to investigate estrogenic effect of any substances, yeast estrogen screening (YES) (Lee *et al.*, 2002), proliferation assay with MCF-7 or E-assay (Strobl and Lippman, 1979) and uterotrophic assay in ovariectomized rats (Benson *et al.*, 1961). It had previously demonstrated that, apart from uterotrophic assay, vaginal epithelium cornification assay was also a reliable tool (Jones and Popes, 1960). The later method was recently applied to demonstrate the difference of estrogenic effect among *P. mirifica* samples collected from 3 provinces in Thailand; Prachuabkirikhan, Saraburi and Chiang Mai (Malaivijitnond *et al.*, 2006).

The proliferative effects on MCF-7 cell cultures were tested against the 5 clones of plant extracts and isoflavonoid standards, in the presence and absence of S9 mixture. The amount of *P. mirifica* that exhibited IC_{50} was >1000 μg/ml. Proliferative effect at low dose (0.1 μg/ml *P. mirifica* extract) in the absence of S9 mixture were found in all *P. mirifica* extracts. The data revealed that at low concentration (0.1 μg/ml) of plant extract acted as a physiological dose for estrogenic effect on ERα-positive cells. The proliferative effect on MCF-7 cell cultures were found in *P. mirifica* collected in summer, PM-II and PM-V at the concentration of 10 μg/ml and PM-I at the concentration of 100 μg/ml. Also, in *P. mirifica* collected in winter, PM-III exhibited proliferative effect on the

same cell culture at the concentration of 100 μg/ml and PM-II showed anti-proliferative effect at concentration of 1000 μg/ml. However, no proliferative effect of *P. mirifica* collected in rainy season on MCF-7 cell cultures was found. The proliferative effects on cell culture were statistic difference in all samples collected in summer and winter seasons. The results implied that the only seasonal, not genetic factor could affect the proliferative effect of plant extract on MCF-7 cell cultures *P. mirifica* extract showed proliferative effect at the low concentrations and anti-proliferative effect at higher concentration (biphasic effect) on ER- positive breast cancer cell line, MCF-7 (Cheewasopit, 2001). The response pattern was similar to phytoestrogen such as genistein and daidzein (Wang and Kruzer, 1997; Zava and Duwe, 1997; Constantinou *et. al.*, 1998; Shao *et. al.*, 2000). Therefore, the results from the experiment could confirm that *P. mirifica* extract contained phytroestrogens.

MCF-7 cells were also treated with plant extracts and standard isoflavonoids, in the presence of S9 mixture. This treatment was an essential test because it represented the imitative metabolism in human consumption. Proliferative effect exhibited in almost every tested dose with the maximum of 10.20, 7.72 and 7.04 folds in *P. mirifica* collected in summer, rainy season and winter, respectively in comparison with the proliferative effect on MCF-7 cells in the absence of S9 mixture. This confirmed that *P. mirifica* phytoestrogens are effectively metabolized by liver enzymes in S9 mixture and potent stronger binding affinity of phytoestrogens to ERα of MCF-7 cells.

In the absence of S9 mixture, there were correlations between 1 μg/ml *P. mirifica* collected in summer with the proliferative effect on MCF-7 cells and the amount of daidzein and genistein. In *P. mirifica* collected in rainy season, there were correlations between 1 and 100 μg/ml *P. mirifica* and the amount of daidzein, correlation between 1 μg/ml *P. mirifica* and the amount of genistein and correlation between 10 μg/ml *P. mirifica* and amount of aglycoside with the proliferative effect on MCF-7 cells. There was no correlation between proliferative effect on MCF-7 and amount of isoflavonoid in *P. mirifica* collected in winter.

In the presence of S9 mixture, the correlations were found between 0.1 and 10 μg/ml *P. mirifica* collected in summer with the proliferative effect on MCF-7 cells and the amount of aglycoside. And there was the correlation between the proliferative effect on MCF-7 cells and ratio of aglycoside/glycoside at 10 μg/ml *P. mirifica*. In *P. mirifica* collected in rainy season, there were correlations between 0.1 μg/ml *P. mirifica* and the

amount of genistin and genistein with the proliferative effect on MCF-7 cells. There was no correlation between proliferative effect on MCF-7 and amount of isoflavonoid in *P. mirifica* collected in winter.

To rank the estrogenic potency of the plant samples by quantitative analysis from this study, using the number of days that the vaginal epithelium became cornified during a sum up number of day during the 14 days of plant material treatment plus the 7 days post-treatment period as a parameter to represent the response of the rat vaginal epithelium submitted to the treatment of *P. mirifica* was obtained.

The number of days that the vaginal epithelium became cornified during a sum up number of day during the 14 days of plant material treatment plus the 7 days post treatment period was recorded. At the dose of 100 mg/kg BW, ovariectomized rats treated with plant samples collected in rainy season showed the least average number (4.40±1.86 days) as compared with summer (11.60±0.25 days) and winter (10.80 ±0.37 days). Nevertheless in treatment of 1000 mg/kg BW *P. mirifica* collected in winter exhibited stronger estrogenic effects (15.00±0.00 days) evaluated from the number of days that the vaginal epithelium became cornified during a sum up number of day during the 14 days of plant material treatment plus the 7 days post treatment period than collected in summer (13.20±0.73 days), and rainy season (13.00±0.32 days). The data confirmed that *P. mirifica* collected in rainy season expressed the weakest estrogenic activity.

There was negative correlation between the numbers of days that the vaginal epithelium became cornified at the dose 100 and 1000 mg/kg BW of *P. mirifica* collected in summer with ratio of aglycoside/glycoside content. The negative correlation between the number of days that the vaginal epithelium became cornified at the dose 100 mg/kg BW of *P. mirifica* collected in rainy season were found with amount of algycoside and the ratio of aglycoside/glycoside content. There was no correlation between the number of days that the vaginal epithelium became cornified and isoflavonoid content in *P. mirifica* collected in winter.

Qualitative analysis of the vaginal cornification was also an additional parameter to evaluate for the different in estrogenic activity of the plants. The percentage of cornified cells at the first day of appearance was chosen to demonstrate this analysis. The results revealed that PM-I, PM-II, PM-III, PM-IV and PM-V in three season exhibited different %cornified cell at the first day of appearance of cornified cells in the dose of

1000 mg/kg BW. Besides, the expression period delayed from the first day in 17β-estradiol treatment to the second to third day of *P. mirifica* treatment. The results suggested that estrogenic activity of *P. mirifica* was lower than 17β-estradiol but it could reach the maximum response as did by 17β-estradiol (100%) once it worked; in all 5 clones of *P. mirifica* collected in winter, PM-III collected in summer and rainy season (93.8, 82.2, 95.6, 85.8, 82.6, 98.4 and 85.8%, respectively). At dose 100 mg/kg BW, the percentage of cornified cells at the first day of appearance (Day 5) was found in PM-III collected in winter (81.6%). The data confirmed that PM-III exhibited the strongest estrogenic activity.

Using uterine weight as parameter, at the concentration of 100 mg/kg BW, *P. mirifica* collected in winter, PM-I, PM-II and PM-V were significantly higher than those from PM-III and PM-IV. However, there were no significant differences of uterine weights in rats treated with 100 mg/kg BW of *P. mirifica* collected in summer and rainy season. The uterine weight in rats treated with 1000 mg/kg BW after treatment period collected in summer and rainy season (0.537±0.031 and 0.478±0.032 g), PM-III was significantly higher than those from the other clones. The uterine weight in rats treated with 1000 mg/kg BW after post-treatment period collected in summer of PM-III (0.471±0.011 g) was significantly higher than those of the others. In rainy season, PM-I was significantly lower than those from the others. However, there was no significant difference of uterine weights in rats treated with 1000 mg/kg BW of *P. mirifica* collected in winter after treatment and post-treatment period. The influence of genetics in *P. mirifica* has been still observed as the higher uterine weights in rats after treated with PM-III collected in summer were found.

There was no significant difference of the mean value of uterine weights treated with 100 mg/kg BW and 1000 mg/kg BW after treatment and post-treatment period among three seasons.

In PM-III collected in summer uterine weights treated with 100 mg/kg BW and 1000 mg/kg BW in treatment and post-treatment period was the most. There was no significant difference of uterine weights treated with 100 mg/kg BW and 1000 mg/kg BW in treatment and post-treatment period of other clones of *P. mirifica*.

Between five clones, in PM-III collected in summer, uterine weights treated with 1000 mg/kg BW after treatment and post-treatment period was significant higher than

those from the other seasons, but there was no significant difference of uterine weights treated with 100 mg/kg BW.

The increment of uterine weights also depended on the dose of treatment, for instance, the uterine weight gain of rats treated with *P. mirifica* at the dose of 1000 mg/kg BW was greater than the treatment with the dose of 100 mg/kg BW. The increment of uterine weights also depended on the clones of *P. mirifica*. The uterine weight gain of rats treated with 1000 mg/kg BW of *P. mirifica* clone PM-III was also greater than those from the other clones.

The uterine weight at the dose 100 and 1000 mg/kg BW *P. mirifica* treated rats was significantly different from the negative and positive controls. This results confirmed uterotrophic effects from the miroestrol (Jones and Pope, 1960) and powder of *P. mirifica* (Malaivijitnond *et al.,* 2004) as claimed previously. Our data was recorded at day 7^{th} after abolish treatment with *P. mirifica*. The uterotrophic response by the plant treatment at 100 and 1000 mg/kg BW should occur but was later diminished maybe because of the rapid secretion of the phytochemicals from the rat bodies according to previous suggestion (Malaivijitnond *et al.*, 2004). Besides, the increment of uterine weight at the end of the post-treatment period should be agreed with the changes of vaginal epithelium cells that were recovered to a stage before treatment with *P. mirifica.*

The plant samples with stronger estrogenic activity could be distinguished at the end of the treatment period and also could maintain more uterotrophic effect, as seen at the end of the post-treatment period. It was found that the uterine weight of PM-III at the end of treatment period in summer was atmost while there was no significant difference in winter. The results were in the same direction as in the analysis of uterine weight at the end of the post-treatment period.

There was negative correlation between the uterine weight treated with *P. mirifica* collected in summer at the dose 1000 mg/kg BW after treatment period with the amount of aglycoside content and the ratio of aglycoside/glycoside. The negative correlations of daidzin and genistin contents were found with the uterine weight treated with *P. mirifica* collected in winter at the dose 100 mg/kg BW in post treatment period. There was no correlation between isoflavonoid contents and the uterine weight treated with *P. mirifica* collected in summer, rainy and winter seasons at the dose of 1000 mg/kg BW in post treatment period.

Qualitative analysis performed after uterus were histologically prepared. The main parameters of analysis were the number of uterine gland and the cross section area. Samples collected in summer, uterine gland number of rats treated with PM-I were more than those from the other clones. Uterine gland number in rats treated with PM-IV collected in rainy season were more than those from the other clones. Samples collected in winter, uterine gland number treated with PM-III was more than those from the others. Significant difference of the mean value of uterine gland number treated with *P. mirifica* collected in rainy season was lower than the ones collected in summer and winter. This finding strongly confirmed that there are seasonal influences on plant estrogenic activity.

The highest uterine gland number treated with PM-I collected in summer was found. But the uterine gland number treated with PM-II and PM-III collected in rainy season were the lowest values. Significant difference of the higher mean value of uterine gland number was found in PM-III.

There was correlation between puerarin contents and uterine gland number in *P. mirifica* harvested in rainy season.

The further analysis was the qualitative analysis of the estrogenic activity. The cross section area of uterine tissue is expressed into 3 parts of the uterus, including myometrium, endometrium and lumen. Samples collected in summer, myometrium and endometrium treated with PM-I was thicker than other clones, lumen treated with PM-III was larger than the others. Myometrium, endometrium and lumen treated with PM-III collected in rainy season were thicker and larger than the other clones. Samples collected in winter, myometrium treated with PM-I, endometrium treated with PM-II was thicker than others. But there was no difference in cross section area of lumen; it meaned that estrogenic effect was not much influence to lumen. There was significant difference of the mean value of myometrium, endometriun and lumen treated with *P. mirifica* collected in summer are thicker and larger than those from the other seasons.

Myometrium treated with PM-I collected in winter was the thinnest, endometrium in summer was the thickest and lumen in rainy season was the largest. Myometrium treated with PM-II collected in rainy season was the thinnest, endometrium in winter was the thickest and lumen in summer is the largest. Myometrium and endometrium treated with PM-III collected in rainy season is the thickest and lumen in summer was the largest. Myometrium treated with PM-IV collected in winter was the thinnest and lumen in winter was the largest. Myometrium treated with PM-V collected in summer was the

thickest, endometrium in rainy season was the thinest and lumen in winter was the largest. There was significant difference of the mean value of myometrium endometriun treated with PM-I were the thicker and lumen treated with PM-III larger than other clones.

There was correlation between cross section area and isoflavonoid contents. The negative correlation between puerarin and aglycoside contents were found with lumen treated with *P. mirifica* collected in summer. There were correlations between myometrium, endometrium and lumen treated with *P. mirifica* collected in rainy season with amount of daidzin. The correlation between daidzein content was found with myometrium treated with *P. mirifica* collected in winter.

In comparison of estrogenic effects initiated by 17β-estradiol versus *P. mirifica* in ovariectomized rats, the treatment exhibited an increase in uterus wet weight in different degree. 17β-estradiol initiated more uterus wet weight than *P. mirifica*. In the analysis of endometrial tissue, it was found that uterus treated with *P. mirifica* showed higher degree of glandular proliferation as well as myometrium and endometrum area while the lumen was smaller. It is a first time of demonstration that phytoestrogen treatment could initiate a better quality differentiation of uterine myometrium and endometrium. Besides, the estrogenic activity found in the differentiation of the uterine tissue is clearer than the result of the cornification analysis. The result also confirmed that phytoestrogen from this plant is not only effective as alteration of estrogen replacement therapy but also more effective. Consider the safety of *P. mirifica* from the toxicity analysis of the plant powder and extract in animals and human volunteers (Cherdshewasart, 2003), it can conclude that *P. mirifica* is the most interesting natural product to be used as phytoestrogen replacement therapy.

The isoflavonoids in tuberous roots collected from 5 clones of *P. mirifica* as the function of seasonal-collected period were analyzed by HPLC. The studies were designed to establish a common environment for all tested plants. Besides, the sample plants were derived from seeds with dormancy and germinated with the same period at the same place then transferred to the same field trial in order to establish a unique age and differentiation of the plants. The results definitely exhibited the influence of both climatic (temperature and amount of rainfall) and genetic factors on isoflavonoid contents in tuberous storage. Consider the climatic factors, the change of rainfall amount plays more important role than the change of temperature. Futhermore, Thailand is in sub-

tropical zone which there is not extreme change in the temperature as much as in temperate zone. Therfore, to harvest the tuber, it has to consider the factors to obtain the high amount of isoflavonoid contents in plant materials.

The results showed that aglycoside (daidzein and genistein) including agycoside/glycoside contents influenced the proliferative effect in MCF-7 cells. There are proliferative effects in MCF-7 cell treated with the *P. mirifica* absence S9 mixture collected summer and winter. The results demonstrated clearly that physical factor exhibited influence on tuberous isoflavonoid storage first which in consequence, influent the estrogenic effect of the chemicals derive from the plant tubers. Besides, genes in isoflavonoid synthesis and/or storage pathway might also play an important role in the presence of isoflavonoid contents in the 5 different plant clones.

The present study provides the first evidence that *P. mirifica* phytoestrogens has profound, dose dependent effect on the vaginal epithelium. The dose of 1000 mg/kg BW exhibits higher estrogenic effect than the dose of 100 mg/kg BW and can effectively stimulate the proliferation of rat vaginal epithelium. Such a plant concentration might be too far from the physiological dose to create a certain amount of binding to estrogen receptor at the vaginal tissue and could not subsequently stimulate the vaginal cornification. At the dosage of 100 mg/kg BW, most of the tested *P. mirifica* samples initiate a significant cornification of the vaginal epithelium as compare with the negative control. The level of response is far less than the initiation of 17β-estradiol in the positive control. At the dosage of 1000 mg/kg BW, 5 cultivars tested samples initiated a greater significant cornification of the vaginal epithelium as compare with the negative control. But this elevated estrogenic response is not in proportion with the added amount of the plant material that is increased in a log scale as compare with the previous dosage. Miroestrol, a key chemical in *P. mirifica* showed equal estrogenic activity to 17β-estradiol in the mouse uterine and to have one quarter of the potency of 17β-estradiol in the rat vaginal cornification test (Cain, 1960). The affinity to ERα and estrogenic response initiation by *P. mirifica* has been already demonstrated in MCF-7, human malignant cell comprising ERα (Cherdshewasart *et al.*, 2004[a]). It is noticed that the cornification response in the combination between ERα and ERβ binding at this dosage is still less than that initiated by 17β-estradiol (81.60%, of cornified cell count of D_5 after treatment with the dose of 100 mg/kgBw of PM-III collected in winter vs. 100% for 17β-

estradiol). It should imply that ERα plays a greater role on cornification of the rat vaginal epithelium than ERβ.

This study is the first report demonstrating the differential estrogenic effect of the cultivated *P. mirifica* collected in three seasons on MCF-7 cell proliferation assay, cornification test, uterotrophic assay, uterine gland number assay and cross section area of uterine tissue assay. The results should benefit in ranking the quality of the tuber-derived materials based on the strength of estrogenic activity in cell culture model, rat vaginal epithelium model, rat uterotrophic response and the changing of uterine morphology. The results demonstrate clearly that there is a differential estrogenic response occurs at the rat vaginal epithelium after treatment with *P. mirifica* derived from different periods of tuber collection.

The result of this study confirmed that influence of climatic and plant genetics on isoflavonoid contents and estrogenic effects will take advantage of cultivated *P. mirifica*. The effective harvest time of *P. mirifica* to accept a full range of phytoestrogen as a phytochemical-source.