國立臺灣大學
生物化學暨分子生物研究所

碩士論文

以 Dabsylation 及 HPLC 系統分析茶的茶胺酸及珈瑪胺基丁酸含量

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碩士論文

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第一部分

利用 Dabsylation 及高效液相層析系統分析茶的

茶胺酸及伽瑪胺基丁酸含量

Analysis of Theanine and GABA contents in Teas (from *Camellia sinensis* L. and *Apocynum venetum* L.) by Dabsylation and HPLC system

第二部分

龍葵葉子水萃物促使 AU565 乳癌細胞進行自噬作用

The water extract of *Solanum nigrum* Linn leaf induced autophagy in AU565 breast cancer cells

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Part I

ABBREVIATIONS USED

Part I

HPLC: high performance liquid chromatography

DABS-Cl: dabsyl chloride; dimethyl-4 aminoazobenzene sulfonyl chloride

E.A.: ethyl acetate

LOD: limit of detection

LOQ: limit of quantification

OPA: o-phthaladehyde

PITC: phenylisothiocyanate

a.a.: amino acid.

Lys: lysine

Asn: asparagine

Arg: arginine

Gln: glutamine

Ser: serine

Glu: glutamic acid

Asp: aspartic acid

Thr: threonine

Thea: theanine

Gly: glycine

Ala: alanine

His: histidine

Tyr: tyrosine

Met: methionine

Pro: praline

Val: valine

GABA: γ-aminobutyric acid

Trp: tryptophan

Phe: phenylalanine

Ile: lsoleucine

Leu: leucine

N.D.: non-detected

Part II

SN leaf: the water extract of *Solanum nigrum* Linn leaf

ATCC: American Type Culture Collection

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal bovine serum

PBS: phosphate buffered saline

SDS: sodium dodecyl sulfate

EDTA: ethylenediamine tetraacetic acid

PMSF: phenylmethylsulfonyl fluoride

BSA: bovine serum albumin

AMPK: AMP-activated protein kinase

mTOR: mammaliam target of rapamycin

c-PARP: cleavage form of poly-ADP-ribose polymerase

MTT: 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl Tetrazolium Bromide

AVO: autophagic vascular organelle

MDC: monodansyl dacarbencine

ROS: reactive oxygen species

DCFH-DA: 2', 7'-dichlorofluorescin diacetate

MMP: mitochondria membrane potential

DiOC6: 3, 3'-dihexyloxacarbocyanine iodide

DAPI: 4',6-diamidino-2-phenylindole

Part I

Analysis of Theanine and GABA contents in Teas (from *Camellia sinensis* L. and *Apocynum venetum* L.) by Dabsylation and HPLC system

1.1.1 中文摘要

Dabsyl chloride (dimethylaminoazobenzene sulfonyl chloride)，在1975年由本實驗室林仁混老師發展而成，可與氨基(amino)及胺類化合物(amine)反應(Dabsylaiton)，被廣泛的使用於胺基酸的分析，在本實驗裡，我們藉由Dabsylation反應及高效液相層析(HPLC)系統在吸光值425nm波長下，分析不同茶，包括綠茶、紅茶、烏龍茶、普洱茶、珈碼茶 (GABA tea) 及羅布麻茶 (luobuma tea) 的茶胺酸(Theanine)、珈瑪氨基丁酸(GABA; γ-aminobutyric acid)及其他胺基酸。在我們的分析中，發現同種茶的茶胺酸不盡相同，產地及製作過程等可能影響此差異，高山茶葉似乎含有較高量的茶胺酸，不論是綠茶還是烏龍茶；普洱生茶(未堆渥發酵)的茶胺酸比普洱熟茶(經堆渥發酵)的茶胺酸多。另外，珈瑪氨基丁酸在一般茶的含量通常不高，除了珈瑪茶，我們發現一些羅布麻茶的珈瑪氨基丁酸含量很高，羅布麻茶含有高量量的珈瑪氨基丁酸是第一次被發現的。

1.1.2 Abstract

Dabsyl chloride (dimethylaminoazobenzene sulfonyl chloride), a useful chromophoric labeling reagent for amino acids and amines, was developed in our laboratory in 1975. Although several methods have been developed to determine various types of amino acids, a quick and easy method of determining theanine, GABA and other amino acids has not been developed in one HPLC system. Here, we analyze the free amino acid contents of theanine and GABA in different teas (green tea, black tea, oolong tea, Pu-erh tea, GABA tea, and luobuma tea) with a dabsylation and reverse phase high performance liquid chromatography (HPLC) system coupled with a detector at 425nm absorbance. In this system, our data suggests that different teas may be recognized by their various theanine contents. The high theanine content of high mountain tea was observed in both green tea and oolong tea. Furthermore, the raw (natural fermented) Pu-erh tea contained more theanine than ripe (wet fermented) Pu-erh tea. And the GABA contents in normal teas were significantly lower than that in Luobuma tea and GABA tea.

1.2 Introduction

1.2.1 Teas *(Camellia sinensis* L.)

Tea is the most popular and widely consumed beverage in the world because of its refreshing taste, attractive aroma, and potential health benefits *(1, 2)*. It has many physiological and pharmacological activities due to the presence of components such as amino acids, polyphenols, carbohydrates, caffeine, purine alkaloids, and vitamins *(3)*. Tea is made from the leaves of the plant *Camellia sinensis* L., which is now widely cultivated in Southeast Asia as well as in several central African countries. Generally, tea can be broadly classified according to the production method as unfermented tea (green tea), half-fermented tea (oolong tea), full-fermented tea (black tea), or post-fermented tea (Pu-erh tea). Green tea and oolong tea are favored in oriental countries, while black tea is favored in western countries.

Pu-erh tea, mainly produced in the Yunnan province of China, is consumed widely in Southeast Asia. Pu-erh tea can be categorized, depending on the way it is piled, as either non-post natural fermented (raw) or post wet fermented (ripe). Both forms of Pu-erh undergo secondary fermentation and oxidization, resulting in a unique type of tea *(4)*.

In Japan, GABA tea also called Gyabaron tea because it is rich in γ-aminobutyric acid (GABA). GABA is an important neurotransmitter with the chief inhibitory in the

mammalian central nervous system. GABA is known to exhibit antihypertensive effects *(5)*, and teas accumulated with GABA have been demonstrated to induce a fall in blood pressure in rats *(6)*. These results demonstrate that GABA has many health-promoting effects, and this has resulted in GABA tea's increasing popularity in recent years.

1.2.2 Luobuma tea (*Apocynum venetum* L.)

Luobuma (Apocynum venetum L.; AV) is a wild plant that grows ubiquitously from central to northwestern China. In contrast to green tea, however, it contains no caffeine, and this has aroused the interest of many researchers regarding its pharmacological activities. Previous papers describe antihypertensive, antihyperlipemic *(7)*, diuretic *(8)*, and antioxidant effects *(9)*. In addition, it has been shown that extracts of AV have anxiolytic-like activity mediated via the GABAergic system *(10)*.

1.2.3 Theanine

Theanine (Glutamic acid γ–ethyl amide; 5-N-ethyl glutamine), as the only free form (nonprotein) amino acid in teas, is very important because of its biological effects and flavorous characteristics. For example, theanine has been demonstrated to

increase serotonin, dopamine, and GABA levels in the brain, which takes part in the neuroprotective effects *(11, 12)*. Moreover, theanine decreases blood pressure in spontaneously hypertensive rats *(13)*. Theanine is also, incidentally, the main component responsible for the exotic taste of tea. The brothy sweet umami taste of green tea is due to amino acids, especially theanine *(14)*.

1.2.4 Methods to detect amino acids

Several methods have been developed to determine the presence of various amino acids. For example, amino acids can be determined in tea by the ninhydrin assay method. High-performance liquid chromatography (HPLC) methods that involve precolumn derivatization with o-phthaladehyde (OPA) *(15)* and phenylisothiocyanate (PITC) *(16)* with fluorescence and diode array UV detection are used for amino acid determination. An improved capillary electrophoretic (CE) separation system and an indirect UV detection system were proposed for amino acid analysis *(17)*. Estimates of dabsyl-amino compounds can be carried out by HPLC with a UV-visible detector at 425nm absorbance, a method previously developed by our laboratory *(18)*.

1.2.5 Dabsyl chloride

Dabsyl chloride (4-dimethylaminoazobenzene-4’-sulfonyl-chloride) is a useful chromophoric labeling reagent for amino acids. It reacts readily with all kinds of amino acids and amino compounds to form chromorphoric dabsyl derivatives, which can be detected on a thin-layer of chromatographic plate and liquid chromatography. The dabsyl-derivatives could be use for the qualitative and quantitative assays of naturally-occurring amines and amino acids because of their stable properties and strong absorbance visibly at 425 nm. In previous studies, the determination of theanine in teas by HPLC with fluorescence and the differentiation of green, white, black, oolong and Pu-erh teas according to free amino acid content have been reported by Alcazar, et al. *(4)*. However, the determination of theanine and GABA with other amino acids by dabsylation has not been developed in one HPLC system. In the present study, we used dabsylation coupled with HPLC systems to determine each free amino acid and focused on analyzing the theanine and GABA content of green, black, oolong, and Pu-erh teas.

1.3 Material and Methods

1.3.1 Chemicals and Reagents.

Standard amino acids, including theanine (Thea) and GABA, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amino acid stock solutions of 1mg/ml were prepared in 0.1M HCl and stored at -20 ℃. Dimethylaminoazobenzene sulfonyl chloride (dabsyl chloride) was also purchased from Sigma-Aldrich. Acetonitrile, and acetone (HPLC grade), were purchased from Romil (Cambridge, United Kingdom). Hydrochloric acid, glacial acetic acid, sodium hydrogencarbonate, and sodium acetate were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system of Millipore (Milford, MA). 1M HCl, 1M sodium hydrogencarbonate (pH 9), and 1mg/ml dabsyl chloride were freshly prepared and dissolved in acetone before dabsylation of samples.

1.3.2 Tea Samples.

Yunnan high mountain tea #1, Yunnan high mountain tea #2, Meng-pa-zhai 2007 (spring), Nanru mountatin 2005, Xi-hu lake Longjing, and Lion mountain Longjing were purchased from China while Ah-Li mountain oolong and Li-shan mountain oolong teas were purchased from Taiwan. Three Pu-erh tea samples were purchased from Yunnan, China. Other commercial tea leaves and tea bags were purchased from a

local tea market in Taipei, Taiwan.

1.3.3 Tea Samples Preparation.

One gram of the tea sample was extracted with 50 ml of hot distilled water (90 ℃) for 30 min. The tea water extract was cooled to room temperature and then filtered through a 0.45 μm nylon filter membrane (Phenomenex). After filtration, the sample's pH was adjusted to 3 by adding 100μl 1M HCl then partitioning with an equal volume of ethyl acetate twice. Water layer pH was adjusted to 9 by adding 2 ml 1M $NaHCO_3$ (pH 9) then partitioning with an equal volume of ethyl acetate once. The water layer containing amino acids was subjected to dabsylation as described below.

1.3.4 Dabsylation of amino acids.

The amino acid solution (1 ml, pH 9.0) was mixed with 1ml dabsyl chloride (1mg/ml, in acetone) and reacted at 67 ℃ for 10 min. The pH of the reactive mixture was kept at 9 by adding 1M $NaHCO_3$ solution. After that, the reaction was stopped at ice-bath, and then the dabsyl-sample was filtered through a 0.45μm nylon filter membrane. When the dabsylation was completed, the free amino acids became dabsyl-amino acids, namely, dabsyl-theanine, dabsyl-GABA, etc. In addition to reacting with amino acids, dabsyl chloride could also react with water to produce

sodium dimethylaminoazobenzene 4-sulfonate (DABS-ONa) as demonstrated by a large and early peak in the HPLC pattern (**Figure 1**, Peak #1).

1.3.5 HPLC Gradient System for Determination of Amino Acids in Tea Samples.

The determination of dabsyl-amino acids derived from tea samples was carried out by HPLC with a spectrophotometric detector at 425 nm as previously described. The HPLC system consisted of a Waters 600E solvent-delivery system, a Model U6K universal injector, and a Jasco UV-visible 975 detector operating at 425 nm. A reversed phase column (Hypersil GOLD Thermo, 250 mm × 4 mm i. d. , 5 μm particle size) coupled with a C18 cartridge was used. The column temperature was maintained at 30 ℃. The composition of the optimized mobile phase A was acetonitrile: 0.045 M CH_3COONa (pH 4) = 30:70. Mobile phase B was acetonitrile: 0.045 M CH_3COONa (pH 4) = 75:25. The gradient elution profile between 0 to 15 min was kept at 100％ of solvent A; 15 to 20 min, linear gradient change to 85％ of solvent A; 20 to 35 min kept at 50％ of solvent A; 35 to 50 min, linear gradient change to 75％ of solvent A; 50 to 65 min, linear gradient change to 0％ of solvent A; 65 to 75 min kept at 0％ of solvent A; 75 to 85 min, linear gradient change to 100 ％ of solvent A. The mobile phase was filtered through a 0.22 μm membrane filter and degassed prior to use. 10 μl of each dabsyl-sample was injected into the

chromatograph column, and the column was ready for the next sample run after column conditioning of more than 30 min.

Another reversed phase column (Zorbax ODS, 250 mm × 4.6 mm i.d. , 5 μm particle size) coupled with a C18 cartridge was used. The gradient elution profile between 0 to 15 min was kept at 100% of solvent A; 15 to 20 min, linear gradient change to 75% of solvent A; 20 to 40 min kept at 75% of solvent A; 40 to 50 min, linear gradient change to 0% of solvent A; 50 to 60 min kept at 0% of solvent A; 60 to 65 min, linear gradient change to 100% of solvent A. The column was ready for the next sample run after column conditioning of more than 30 min. Other parameter of this system is the same as Hypersil GOLD Thermo column described above.

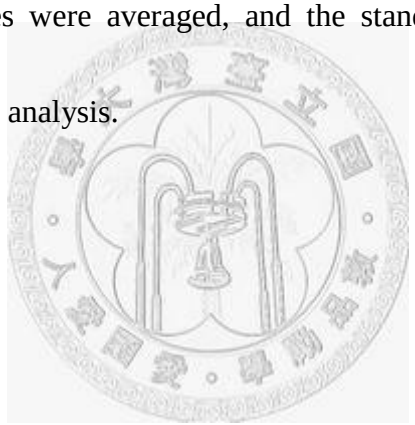
1.3.6 HPLC Method Validation.

In this system, the precision, selectivity, accuracy, detection quantification limits, and linearity ranges were estimated. To evaluate the precision of the method, five replicate analyses of a standard solution on different days were performed for both the retention time and the peak area of the dabsyl-amino acid standards. The resolutions were more than 0.5 mins for all of the determined peaks beside peaks 1 and 2. The accuracy was assessed by recovery experiments. Known amounts of dabsyl-amino acid standards were added to the tea dabsylated-sample. The recovery rate was

calculated by comparing the obtained amounts with those added, and their values ranged between 90 to 100%. Calibration curves were constructed over six different concentrations. Each standard was analyzed in triplicate, and the peak area was plotted against the corresponding concentration.

1.3.8 Data Analysis.

For all of the measurements, more than three replicate samples were taken for analysis. All of the values were averaged, and the standard deviation (S.D.) was obtained for statistical data analysis.



1.4 Results

1.4.1 Condition description

The separation of standard dabsyl-amino acids is shown in **Figure 1**. By the reversed phase column (Hypersil GOLD Thermo), the dabsyl free amino acids derived from Ser, Thr, Thea, Gly, Ala, Val, GABA, Trp, Phe, Ile, Leu, Tyr could be detected in base-line separated peaks on the chromatogram (**Figure 1a**). A better separation of standard dabsyl amino acid was achieved by the Zorbax ODS column (**Figure 1b**), that the dabsyl Asn, Gln, Ser, Asp, Glu, Arg, Thr, Thea, Gly, Ala, Met, Pro, Val, GABA, Trp, Phe, and Tyr could be detected separately on the chromatogram (**Figure 1b**). Chromatograms of the tea samples analyzed by Hypersil GOLD Thermo column are shown in **Figure 2a-g,** including those of green, oolong, black, Pu-erh, GABA tea, and Luobuma tea. The quantitative pattern of amino acids, particularly of theanine and GABA is shown in **Table 1** to **Table 3**. The presence of theanine and GABA were confirmed by co-chromatography with an authentic standard. Under these conditions, the calibration curves for measuring both dabsyl-theanine and dabsyl-GABA showed a coefficient of linearity near unity ($R^2 = 0.99$). The lower limit of quantification (LLOQ) was more than 1000 ng while the limit of detection (LOD) was 11.62 ng (for dabsyl-theanine) and 3.43 ng (for dabsyl-GABA), respectively. By the manufacturing method, tea samples were divided into three groups, green, oolong,

and other teas.

1.4.2 Green tea

Table 1, first group, shows the theanine content of green tea which varied from 686.53 to 3029.98 μg/g. After sub-classifying green tea into three groups—general tea leaves, high mountain tea leaves, and the powder of tea bags—the variance of theanine content became 686 to 936 μg/g in the powder of tea bags, but 1307 to 3029 μg/g in general and high mountain tea (**Figure 3a**). The GABA contents were low on average, only Ah-Li mountain oolong tea and Zhen-pin Longjing tea reached to 90 μg/g.

1.4.3 Oolong tea

In the second group, the theanine content of oolong tea varied from 170.06 to 2831.40 μg/g (**Table 2**). After sub-classification of oolong tea into three groups, the theanine content varied from 406.24 to 744.30 μg/g in Dong Ding oolong tea, 2430.34 to 2831.40 μg/g in high mountain oolong tea, and 170.06 to 1677.74 μg/g in Tenren oolong tea. Interestingly, the theanine content in the Tenren oolong tea group correlated to its sale price and quality. **Figure 3a** illustrates that oolong tea samples show significant amounts of theanine present by their source, and these results

indicate high mountain tea contains a higher level of theanine. The GABA contents were low, aside from that of Lisan high mountain oolong tea. Like Zhen-pin, Lisan high mountain oolong tea contains high theanine and GABA contents. Take together, the theanine content in high mountain green and oolong tea averaged more than 2000 μg/g (**Figure 3b)**.

1.4.4 Black, Pu-erh, GABA tea, and Luobuma tea

Black tea, Pu-erh tea, GABA tea, and Luobuma tea were involved in the third group. In black tea, the theanine content varied from 470.77 to 1070.19 μg/g, and their GABA contents were all below 55.45 μg/g (**Table 3**). The sub-classified Pu-erh teas were Pu-erh ripe tea and Pu-erh raw tea. For Pu-erh ripe tea, neither theanine nor GABA content could be detected. In contrast, the theanine content was from 862.95 to 1054.49 μg/g and the GABA content was about 45 μg/g in Pu-erh raw tea. Compared to the green tea, Yunnan high mountain tea #1 and Yunnan high mountain tea #2 are also sources of leaves for making Pu-erh tea. The theanine content decreases in the following order: Yunnan high mountain green tea, Pu-erh raw tea and Pu-erh ripe tea (**Figure 3c**). GABA teas has high GABA content, manufactured as they are by a special procedure under anaerobic conditions. The commercial GABA tea sample contained less theanine (237.94 μg/g) and more GABA (197.51 μg/g) compared to

green teas. In addition, luobuma tea showed a significantly higher level of GABA than other teas (**Figure 3d**).

1.5 Discussion

1.5.1 The advantage of dabsyl chloride methods

Dabsyl chloride can react with the amine group to form a stable product like dabsyl amino acid. Dabsyl products with a strong absorbance at 425 nm can be applied to couple with TLC or HPLC separate system *(19)*. Compared to o-phthaladehyde (OPA) or phenylisothiocyanate (PITC), dabsyl a. a. is relatively stable and can be stored for more than one month at 4 ℃. Moreover, the detection limit of dabsyl theanine (11.62 ng) is significantly better than that of OPA (120 ng) *(20)*. Furthermore, the lower limit of quantification of dabsyl theanine can be more than 1000ng. Incidentally, Pro and Pro-OH are secondary amino acids that can not directly react with PITC or OPA reagents *(21)*. However, they can react with dabsyl chloride directly by the dabsyl method. In this study we demonstrated our HPLC system is suitable for the analysis of dabsyl Ser, Thr, Thea, Gly, Ala, Val, GABA, Trp, Phe, Ile, Leu, Tyr, separately. In particular, we focus on determining the contents of theanine and GABA because of their potential health benefits.

1.5.2 Theanine

Theanine is a unique free amino acid found almost exclusively in tea plants. It is the main component responsible for giving tea its taste and possesses some

pharmacological activities. For these reasons, theanine is usually used as an index for determining the characteristics and quality of teas *(14)*. Alcazar et. al. have shown amino acid patterns in teas; however, the theanine content of various teas were diverse, even in the same kind of tea *(4)*. In the presented data in this paper, we tried to explain the theanine diversity in some teas. When extracted from green and oolong tea leaves (**Figure 3b)**, the highest theanine is found predominately in high mountain teas, in general teas, and then in tea bags. High level of theanine in the high mountain tea and low level in the tea bag, these partially explained theanine content diversity may due to the sample source. In Pu-erh teas, as shown in **Figure 3c**, the theanine of Yunnan high mountain green tea (1500 μg/g) exceeded that of Pu-erh raw tea (900 μg/g), which in turn exceeded that of Pu-erh ripe tea (N. D.). Yunnan high mountain green tea is made with steps similar to Pu-erh raw tea, but without the secondary fermentation and oxidization. The difference between the Pu-erh raw and ripe teas was principally in the piling steps. The disparity of tea making steps may explain the theanine content diversity in Pu-erh teas.

1.5.3 GABA

Because of the hypotensive effect of GABA, research on the GABA level in teas has been potentially attracted. The accumulation of GABA contents under anaerobic

conditions can be observed in many plants, and the technique for arising GABA content in teas has been well established *(22, 23)*. Furthermore, it has been demonstrated that the growth condition of plants and the manufacture process may affect GABA content. In our samples, besides GABA tea, only luobuma tea had a higher GABA content while other teas seemed to have a lower GABA content (less than 100 μg/g). To the best of our knowledge, the high GABA content in luobuma tea was evaluated here for the first time.

1.5.4 Problems and resolved

Because dabsyl chloride reacts with primary amines, secondary amines, and some hydroxyl groups, it is a powerful reagent for the microdetermination of amino acids, aliphatic amines, and polyamines *(18)*. Free amino acids, like His, Lys, and Tyr have more than one functional group, which means they could form multiple derivatized products. Separating these products from other dabsyl amino acids could prove challenging. This problem, however, could be solved because in tea sample dabsylation, dabsyl chloride is a major reactant and amino acid content is relatively low, so that the dabsyl amino acid would be the major “dabsyl-saturated” product, as demonstrated by the fact that the major peak of Tyr is peak 17. Therefore, peaks 6, 8 and 10 could be taken as Thea, Ala and Val.

1.5.5 Summary

In summary, we focus on determining the theanine and GABA contents in teas by a dabsylation HPLC system. We found that the theanine viability in green and oolong tea samples may be partially due to the sample source. High mountain teas usually contain high levels of theanine. Second, different tea making steps, like those employed with Pu-erh teas, may contribute to the theanine diversity. Finally, luobuma tea seems to contain a high level of GABA.

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1.7 Figures

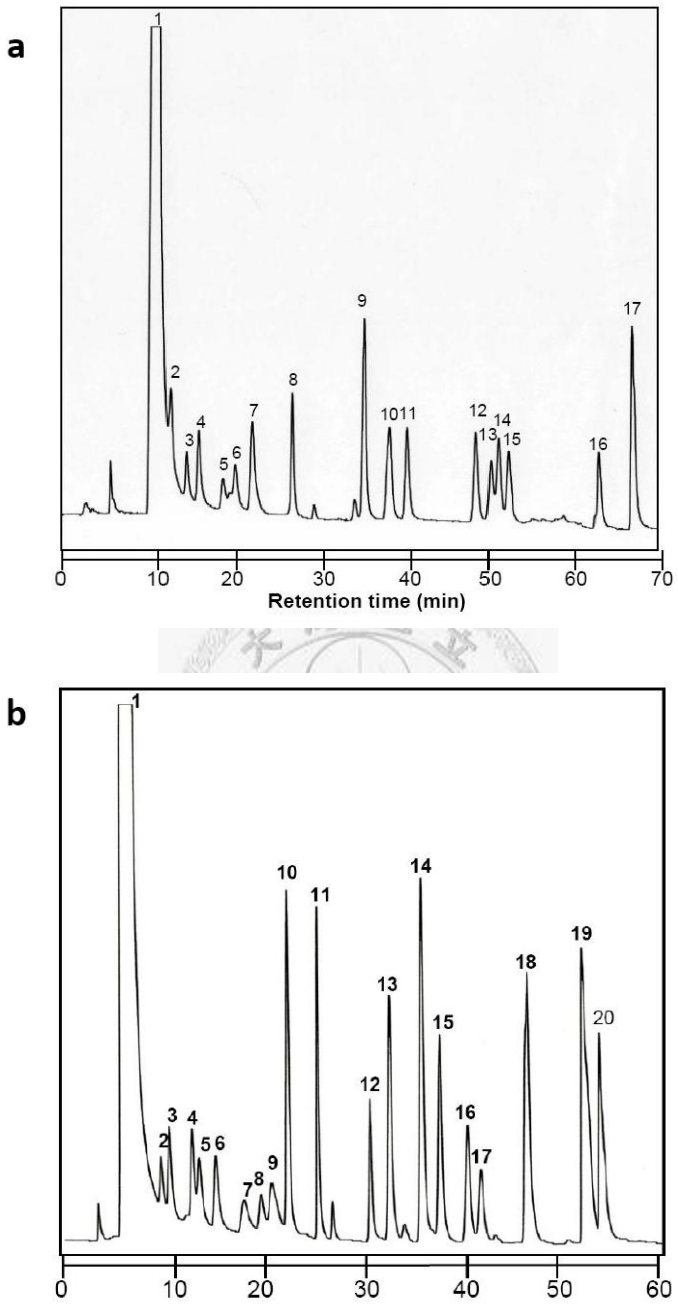


Figure 1. Representative HPLC patterns of standard dabsyl-amino acids by gradient elution and detected at 425nm. Figures illustrate typical RP-HPLC chromatograms of authentic dabsyl free amino acids and the order of elution by retention time. (a) Hypersil GOLD Thermo column: 1. DABS-ONa + Asn; 2. Arg + Gln; 3, Ser; 4, Glu + Asp; 5, Thr, 6, Thea; 7, Gly; 8. Ala; 9, Met + Pro; 10, Val; 11, GABA; 12, Trp; 13, Phe; 14, Ile; 15, Leu; 16, His + Lys and 17, Tyr. (b) Zorbax ODS column: 1. DABS-ONa + Cys; 2. Asn; 3, Gln; 4, Ser; 5, Asp; 6, Glu; 7, Arg; 8. Thr; 9, Thea; 10, Gly; 11, Ala; 12, Met; 13, Pro; 14, Val; 15, GABA; 16, Trp; 17, Phe; 18, Ile + Leu; 19, His + Lys; 20,Tyr.

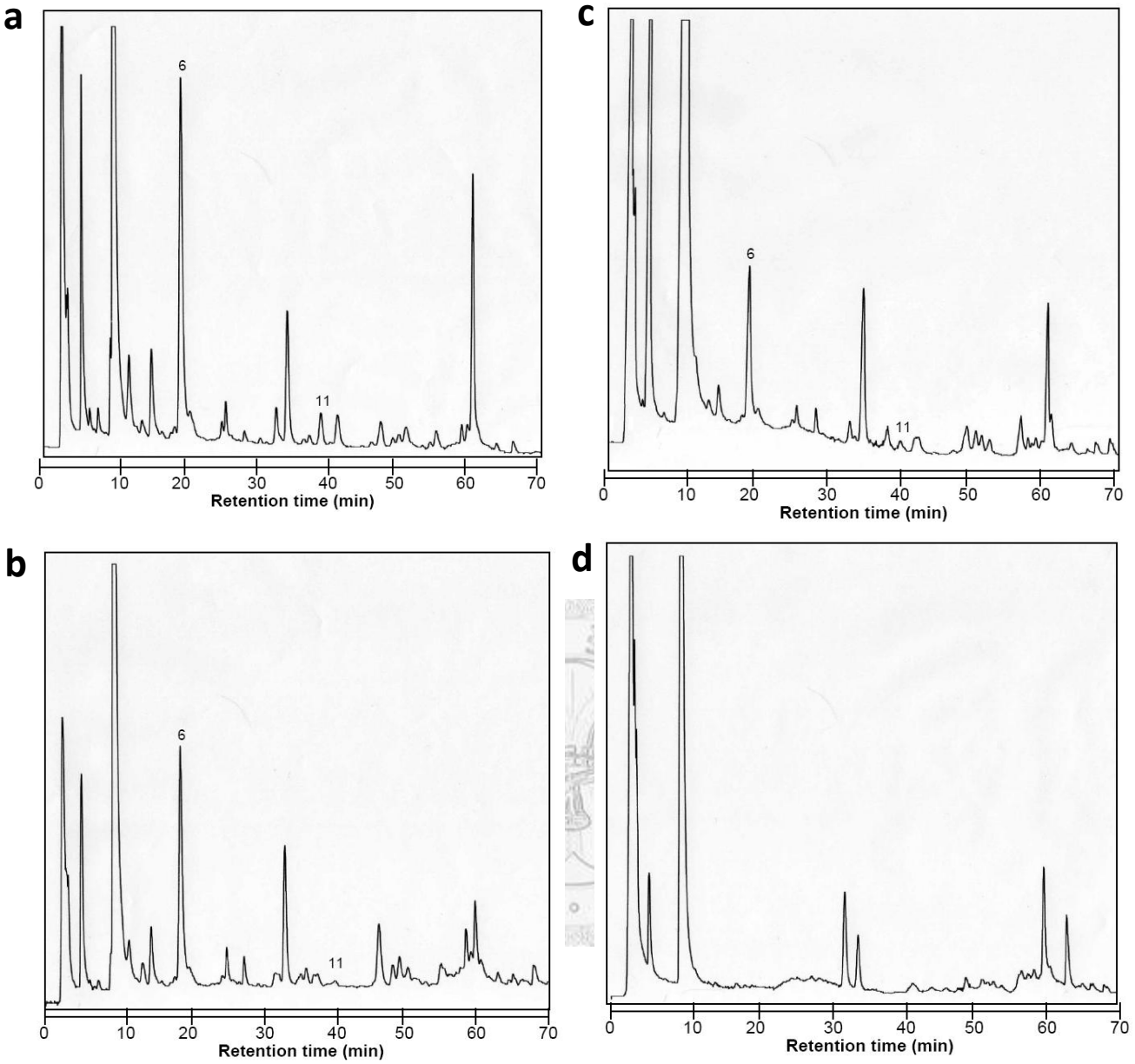


Figure 2. The represented HPLC patters of dabsyl amino acids including theanine and GABA contents in various teas, including green tea (a), oolong tea (b), black tea (c), Pu-erh ripe tea (d) as indicated. The numbers 6 and 11 indicate theanine and GABA, respectively. Samples were derivatized with dabsyl chloride, and detection was at 425 nm. Hypersil GOLD Thermo column.

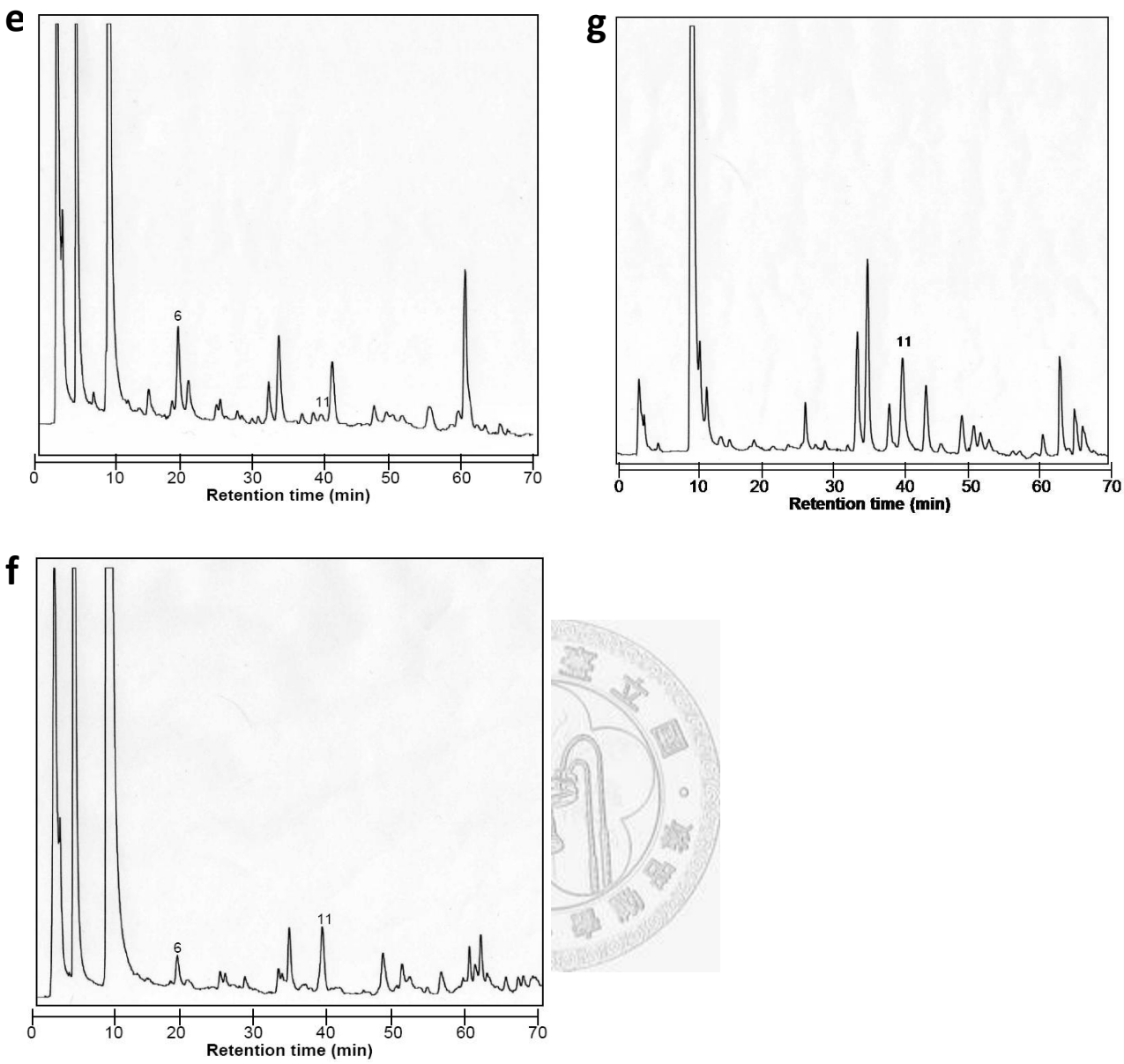


Figure 2. The represented HPLC patters of dabsyl amino acids including theanine and GABA contents in various teas, including Pu-erh raw tea (e), GABA tea (f), and Luobuma tea as indicated. The numbers 6 and 11 indicate theanine and GABA, respectively. Samples were derivatized with dabsyl chloride, and detection was at 425 nm. Hypersil GOLD Thermo column.

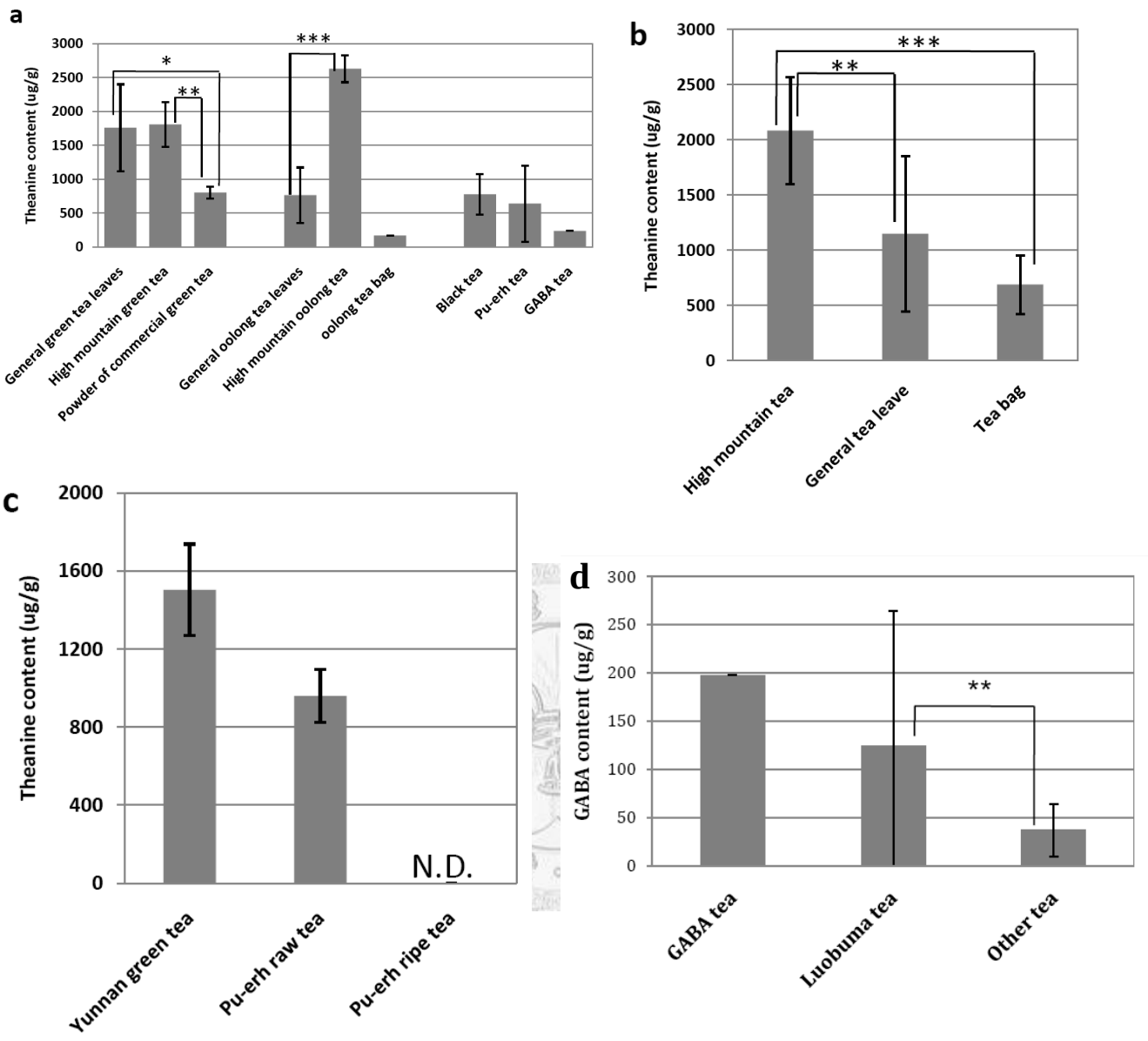


Figure 3. Comparison of theanine and GABA contents in various teas. (a) Average theanine contents from teas categorized by piling steps as non-fermented teas (green tea), semi-fermented teas (oolong tea), and other teas (black tea, Pu-erh tea, GABA tea and luobuma tea). (b, c) Comparison of theanine contents by tea manufactory source and procedures. (d) Comparison of GABA contents in various teas. Lobuma tea has the most abundant GABA contents compared to other teas. Bar depicts mean ± SE of each group. *, ** and *** represent a statistically significant between difference groups, $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

1.8 Tables

Table 1. Quantification of Theanine, GABA, and other amino acid Contents in Green Tea.

Name	Theanine[c]	GABA	Ser	Thr	Gly	Ala[d]	Val[e]	Trp	Phe	Ilu	Leu	Tyr[f]
General green tea leaves												
Xi-hu lake Longjing, China	1307.86 ± 99.57 [a]	53.71 ± 7.81	86.85	N.D.	N.D.	42.48	N.D.	26.73	12.67	16.81	66.20	5.34
Tenren, green tea (Longjing), Taiwan	1400.90 ± 81.58	26.35 ± 7.35	135.03	N.D.	N.D.	47.43	N.D.	36.11	26.37	33.06	34.50	6.93
Qingxin-biluo (Min-lao tea factory)	1492.96 ± 64.87	90.78 ± 17.39	199.90	186.35	49.78	93.153	254.23	95.52	147.07	137.74	103.98	5.23
Lion mountain Longjing , China	1562.85 ± 70.96	68.43 ± 9.80	N.D.	N.D.	N.D.	N.D.	45.22	8.46	18.91	74.65	N.D.	4.38
Zhen-pin (High grade) Longjing, China	3029.98 ± 171.58	105.40 ± 9.94	176.50	105.33	35.32	85.40	51.56	51.60	12.78	15.04	74.20	13.3
High mountain green tea												
Yunnan high mountain tea #1, China	1336.75 ± 79.70	58.28 ± 13.04	112.50	N.D.	N.D.	58.49	74.78	25.47	30.02	22.20	39.24	4.32
Yunnan high mountain tea #2, China	1668.44 ± 72.58	52.07 ± 10.32	87.59	N.D.	N.D.	42.52	71.19	N.D.	N.D.	N.D.	N.D.	5.97
Meng-pa-zhai (2007 spring), Yunnan,China	2111.38 ± 67.57	N.D.[b]	91.62	N.D.	N.D.	58.07	70.82	71.00	76.04	17.60	28.02	8.61
Nanru mountatin (2005 spring), China	2116.52 ± 85.77	N.D.	88.13	N.D.	N.D.	38.12	N.D.	N.D.	N.D.	N.D.	N.D.	9.77
Powder of commercial green tea												
Tenren, green tea powder, high grade,Taiwan	686.53±75.85	20.56±7.82	163.16	N.D.	N.D.	36.84	127.55	15.02	30.51	31.05	35.62	3.38
Tenren, green tea powder, Taiwan	786.85±75.37	19.83±8.28	142.28	N.D.	N.D.	29.73	71.43	47.71	11.24	51.49	37.37	2.42
Anxin, green tea powder	791.47±74.24	31.70±7.62	137.26	N.D.	N.D.	N.D.	95.86	74.81	27.47	54.16	38.12	9.97
Tenren, cold steep green tea powder	936.15±80.13	19.61±8.09	136.40	N.D.	N.D.	37.13	45.88	18.01	24.94	63.14	49.01	6.68

[a]value (ug/g)=mean ± S.D. (n≧3), [b]N.D., non detectable, [c]peak6=Thea (major) + Lys (minor), [d]peak8=Ala (major) + His (minor), [e]peak10=Val (major) +Tyr (minor), [f]peak17=Tyr (major).

Table 2. Quantification of Theanine, GABA, and other amino acid Contents in Oolong Tea.

Name	Theanine[c]	GABA	Ser	Thr	Gly	Ala[d]	Val[e]	Trp	Phe	Ilu	Leu	Tyr[f]
Tenren oolong tea												
Tenren Oolong tea bag	170.06±63.03[a]	19.17±7.58	140.68	N.D.	N.D.	N.D.	80.86	26.39	34.56	57.42	50.47	5.32
Tenren Oolong (low quality)	699.86±67.67	19.97±7.51	102.12	N.D.	N.D.	N.D.	51.62	30.33	18.97	25.28	35.18	4.68
Tenren Oolong (middle quality)	1130.43±81.08	23.95±8.16	187.47	N.D.	N.D.	48.96	72.30	63.08	51.38	39.88	31.46	9.39
Tenren Oolong (high quality)	1677.74±69.92	34.61±8.21	189.59	N.D.	N.D.	60.43	80.19	45.08	52.76	54.96	46.50	7.99
Dong Ding oolong tea												
Nantou Deer valley, Dong Ding	406.24±73.05	25.79±7.67	133.52	N.D.	N.D.	28.68	N.D.	43.92	27.37	58.02	34.54	5.32
Green hill tender leave tea (super)	441.90±75.78	31.16±7.68	144.45	N.D.	N.D.	37.57	45.37	18.85	72.72	46.60	30.23	10.36
Hua-tai tender leave tea	466.88±75.20	31.32±7.38	102.50	N.D.	N.D.	N.D.	59.90	33.06	36.17	45.73	35.33	8.27
Tenren, Dong Ding Oolong Tea	553.63±72.87	39.78±7.97	140.68	N.D.	N.D.	33.81	45.86	26.39	34.56	57.42	50.47	5.77
Dong Ding spring tea	744.30±69.25	N.D.[b]	110.59	N.D.	N.D.	N.D.	N.D.	34.25	35.26	34.60	59.42	7.91
High mountain oolong tea												
Ah-Li mountain oolong tea	2430.34±147.41	46.29±9.82	104.10	125.54	44.02	79.59	97.83	52.51	27.77	57.80	34.74	14.33
Lisan high mountain oolong tea	2831.40±265.89	101.16±11.57	104.28	N.D.	44.94	31.81	47.30	28.11	13.28	37.83	27.90	5.52

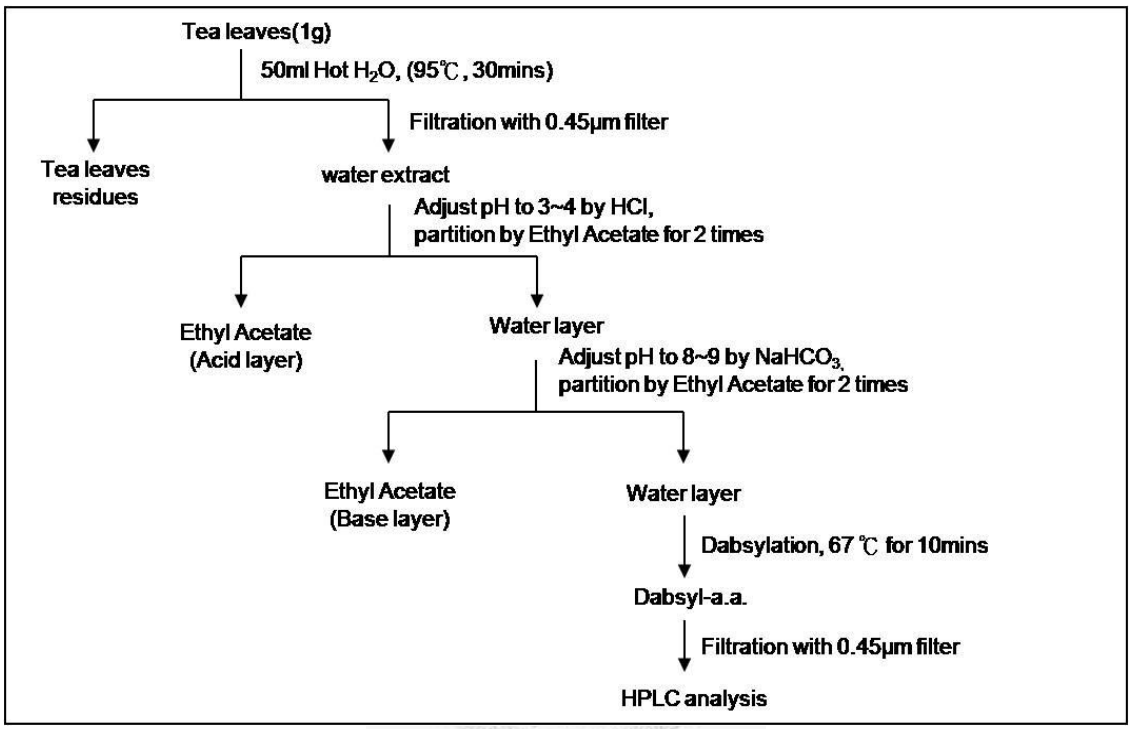
[a]value (ug/g)=mean ± S.D. (n≧3), [b]N.D., non detectable, [c]peak6=Thea (major) + Lys (minor), [d]peak8=Ala (major) + His (minor), [e]peak10=Val (major) +Tyr (minor), [f]peak17=Tyr (major).

Table 3. Quantification of Theanine, GABA, and other amino acid Contents in Black Tea, Pu-erh Tea, GABA Tea, and Luobuma Tea.

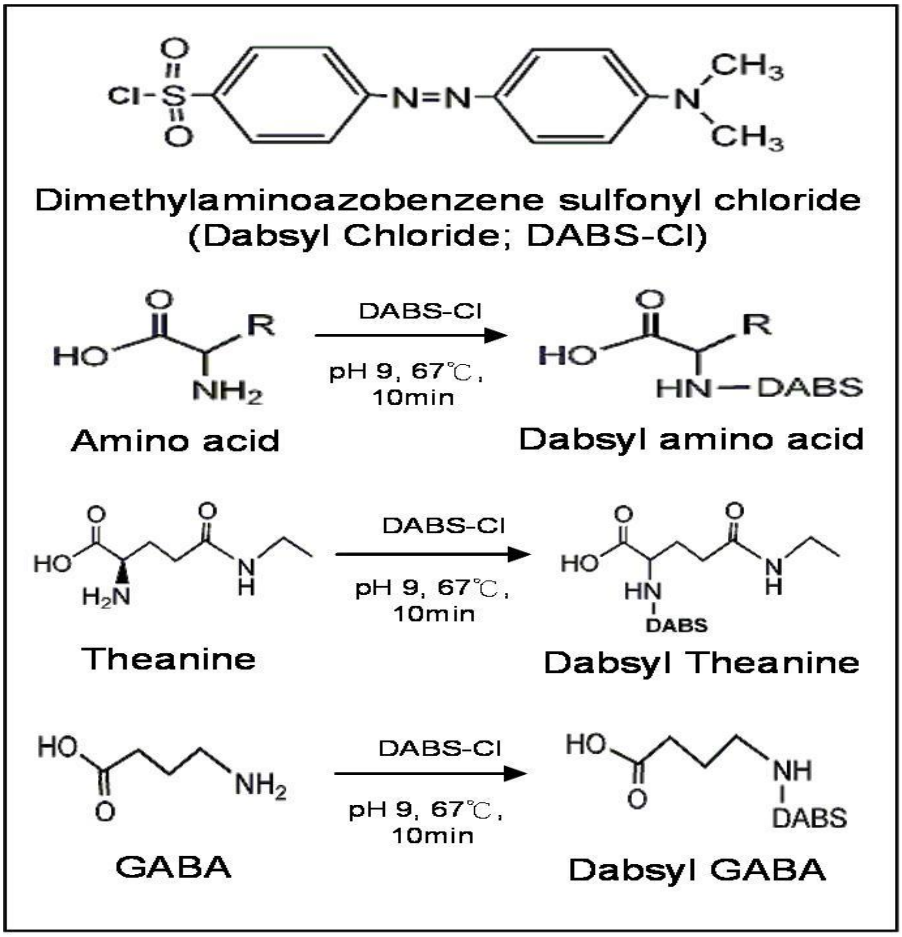
Name	Theanine[c]	GABA	Ser	Thr	Gly	Ala[d]	Val[e]	Trp	Phe	Ilu	Leu	Tyr[f]
Black tea												
Stassen pure ceylon black tea	470.77±72.74[a]	55.45±10.11	113.50	N.D.	N.D.	N.D.	N.D.	81.29	27.37	58.02	36.37	6.32
London black tea	784.87±64.26	N.D.[b]	87.55	N.D.	N.D.	16.52	64.17	23.18	30.12	22.96	31.81	10.47
Lipton yellow label black tea	1070.19±95.36	34.50±8.10	119.46	N.D.	N.D.	33.90	74.84	83.45	71.78	48.81	54.69	7.20
Pu-erh tea												
Pu-erh ripe tea 2004,wet fermentation,Yunnan, China	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.59
Pu-erh raw tea, Yi-Wu main mountain,China	862.95±69.53	44.56±11.90	115.73	N.D.	N.D.	46.35	54.40	8.44	16.39	12.90	31.37	6.33
Pu-erh raw tea 2004, natural fermentation.Yunan, China	1054.49±71.56	46.07±18.16	86.01	N.D.	N.D.	12.87	63.02	6.43	16.42	15.04	31.37	4.12
Special made tea												
GABA tea	237.94±73.69	197.51±8.40	N.D.	N.D.	N.D.	N.D.	56.17	75.18	25.24	55.06	35.16	5.44
Luobuma tea												
Luobuma tea # 1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Luobuma tea # 8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Luobuma tea # 4	N.D.	36.31±25.66	N.D.	N.D.	N.D.	N.D.	67.31	27.93	23.38	29.21	41.87	27.94
Luobuma tea # 3	N.D.	41.70±9.08	N.D.	N.D.	N.D.	N.D.	87.64	31.35	25.69	26.69	35.23	31.35
Luobuma tea # 7	N.D.	43.69±11.94	N.D.	N.D.	N.D.	N.D.	67.29	35.95	31.37	30.45	29.72	34.19
Luobuma tea # 5	N.D.	269.38±19.42	69.84	N.D.	N.D.	73.38	179.45	73.92	61.38	31.09	30.30	92.24
Luobuma tea # 6	N.D.	287.84±21.39	78.32	N.D.	N.D.	67.93	138.31	68.33	33.94	40.92	33.24	70.12
Luobuma tea # 2	N.D.	319.79±15.41	88.81	N.D.	N.D.	91.89	205.32	76.42	71.36	41.09	38.28	76.42

[a]value (ug/g)=mean ± S.D. (n≧3), [b]N.D., non detectable, [c]peak6=Thea (major) + Lys (minor), [d]peak8=Ala (major) + His (minor), [e]peak10=Val (major) +Tyr (minor), [f]peak17=Tyr (major).

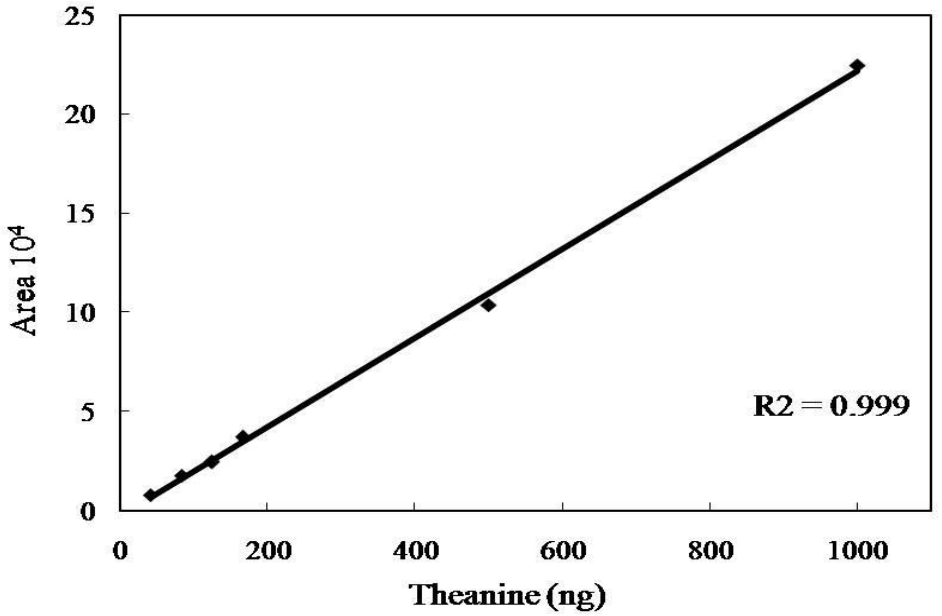
1.9 Appendix

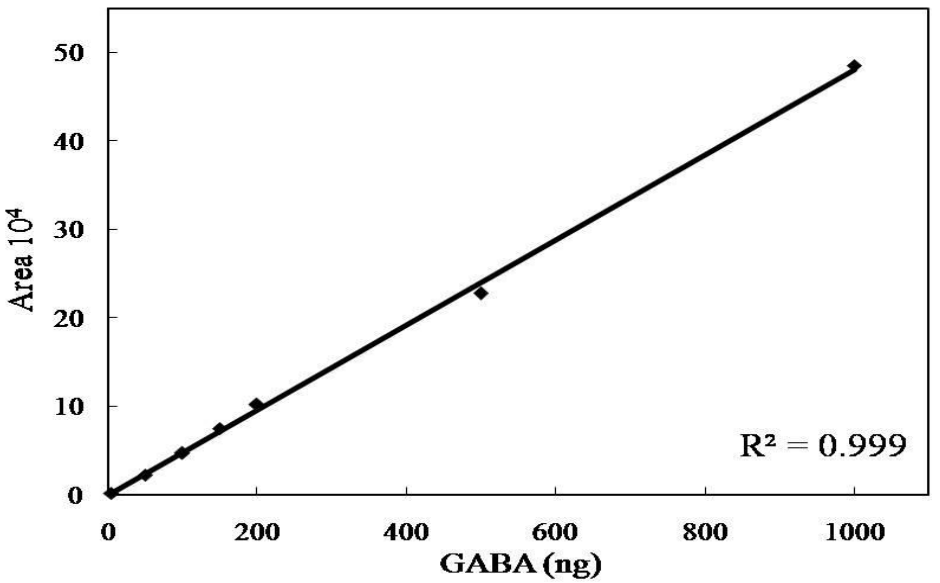


Appendix 1. Experimental procedure for dabsylation of amino acids from tea samples.

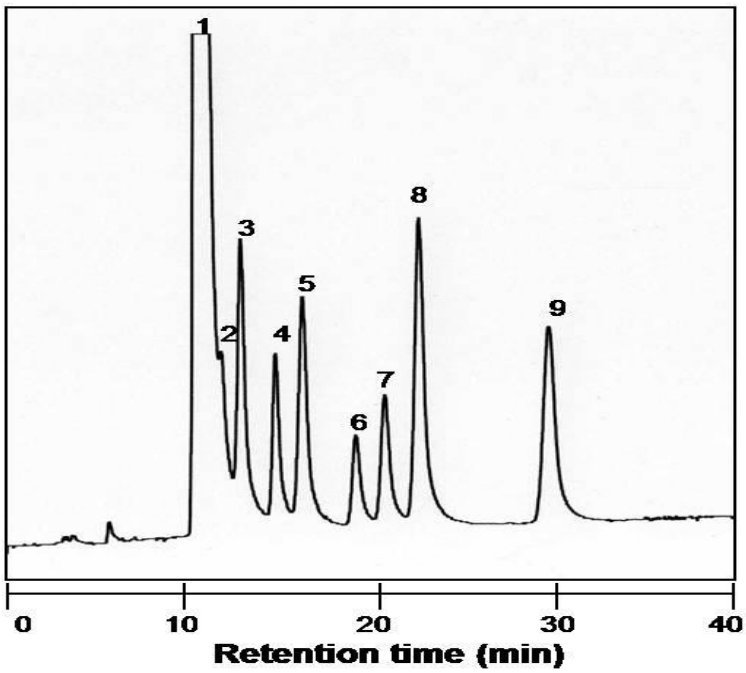


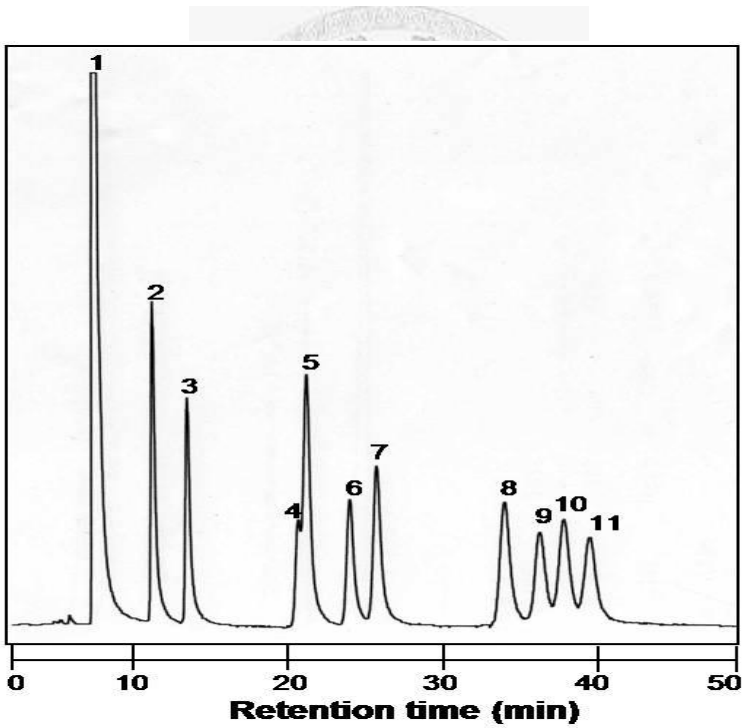
Appendix 2. The structure formula of dabsyl chloride and dabsylation of amino acids.





Appendix 3. Calibration curves for measuring dabsyl- theanine and-GABA at 425 nm.

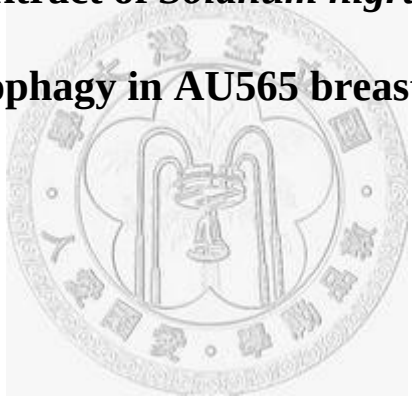




Appendix 4. Isocratic separation of dabsyl amino acids. (a) For the determination of dabsyl theanine. Mobile phase; Acetonitrile : 0.045M sodium acetate (pH 4) = 30:70 (v/v). Peaks: 1. DABS-Ona + Cys; 2, Asn;3, Arg+ Glu; 4. Ser; 5, Glu + Asp; 6, Thr; 7, Theanine(Thea); 8. Gly; 9, Ala-His-I (b) For the determination of dabsyl GABA. Mobile phase, Acetonitrile: 0.045 M sodium acetate (pH 4) = 35:70 (v/v). Peaks: 1. DABS-Ona; 2.Gly; 3. Ala+His-I, 4.Met; 5, Pro; 6, Val + Tyr-II; 7. GABA; 8. Trp; 9, Phe; 10, Ile;11, Leu. Column: Hypersil GOLD Thermo.

Part II

The water extract of *Solanum nigrum* Linn leaf induced autophagy in AU565 breast cancer cells



2.1.1 中文摘要

Solanum nigrum Linn,中文又稱為龍葵、苦葵、黑甜仔菜，為茄科茄屬植物，可在亞洲溫帶季候區之田野中發現，一般傳統中草藥描述其性味苦寒有小毒，具利水消腫、清熱解毒及保肝之功效，然而龍葵的藥用機轉乃須更進一步的探討與研究。在我們實驗室，我們使用龍葵葉子的水萃物，以 MTT 比較龍葵對不同細胞的效應，包括肝癌細胞、乳癌細胞、及纖維組織母細胞，發現 AU565 乳癌細胞較具敏感性。龍葵水葉子萃物引發 AU565 進行自噬作用而非細胞凋亡，其作用機轉似乎與 Akt 或 AMPK 路徑的調控無關，低濃度(< 25 μg/ml)的龍葵葉子水萃物引起之自噬作用並無法使得細胞死亡，可能是因為自噬作用被後期增加之 p-Akt 所抑制，高濃度(> 100 μg/ml)之龍葵葉子水萃物可抑制此增高之 p-Akt 進而促使細胞死亡，這些結果再一次確認了 Akt 對於 AU565 癌細胞之重要，而龍葵水萃物對於治療 AU565 相關之癌症，仍需進一步評估。

2.1.2 Abstract

Solanum nigrum Linn belong to *Solanaceae* is a plant widely grows in south Asia and has been used in traditional folk medicine. It is believed to have many biology activities including antipyretic, diuretic, anti-cancer, and hepatoprotective effect. However, the exactally mechanism of such effects needed further research. In our lab, we used the water extract of *Solanum nigrum* Linn leaf (SN leaf) to compare the response of different cells, including liver cancer, breast cancer, and fibroblast. The results showed that AU565 bresat cancer cells were more sensitive to the extract. SN leaf induced autophagy but not apoptosis in AU565. The induced autophagy seemed not related to Akt or AMPK pathway, but the exactly mechanism was still unknown. Low dose (< 25 μg/ml) of SN leaf induced autophagy would not induce cell death because of the raising of p-Akt to inhibit autohpagy. Higher dose (> 100 μg/ml) of SN leaf could inhibit the level of p-Akt and cause the cell death. These confirmed that Akt is the key survival factor for AU565, and the using of SN leaf for the treatment of AU565 related cancers were needed further evaluation.

2.2 Introduction

2.2.1 Breast cancer

2.2.1.1 Death rate

Breast cancer is by far is the second most common type of cancer after lung cancer (10%, both sexes), the most commonly diagnosed cancer (21%) and the second-most common cause of cancer death (after lung cancer) in women worldwide *(1)*. In the U.S. women have a 1 in 8 (12.5%) lifetime chance of developing invasive breast cancer and a 1 in 35 (3%) chance of breast cancer causing their death (about 7% of cancer deaths; almost 2% of all deaths) *(2)*.The five year survival rate of women with advanced stage breast cancer is still only 20% *(3)*. Such high mortality due to breast cancer is associated primarily with late detection and recurrent disease *(4)*.

2.2.1.2 Etiology

No etiology is known for 95% of breast cancer cases, while approximately 5% of new breast cancers are attributable to hereditary syndromes. Like other forms of cancer, breast cancer is considered to be the final outcome of multiple environmental and hereditary factors. Mutations that can lead to breast cancer have been experimentally linked to estrogen exposure *(6)*, abnormal growth factor signaling,

failure of immune surveillance, and inherited DNA repair genes defects, such as *BRCA1*, *BRCA2 (7)* and *p53*.

Many epidemiological risk factors have been identified. The number of cases worldwide has significantly increased since the 1970s, a phenomenon partly blamed on modern lifestyles. There were substantial variation in breast cancer rates from higher industrialized countries to developing countries; with high rates in North America and Western Europe, intermediate rates in South America and Eastern Europe, and low rates in Asia *(1)*. Other risk factors that have been identified are sex *(5)*, age, obesity, childbearing, hormones *(9)*, growth factors, alcohol intake *(8)*, a high-fat diet *(10)*, tobacco use, and radiation.

Additional, younger women tend to have a poorer prognosis than post-menopausal women due to several factors. Their breasts are active with their cycles, they may be nursing infants, and may be unaware of changes in their breasts. Therefore, younger women are usually at a more advanced stage when diagnosed. And breast cancer is also occurs in males because breast is composed of identical tissues in both males and females *(5)*. The incidences of breast cancer in men are approximately 100 times less than in women. But men with breast cancer seem to have the same statistical survival rates as women. Actually, in the U.S., both incidence and death rates for breast cancer have been declining in the last few years. However,

breast cancer remains the most feared disease.

2.2.1.3 Treatment

The mainstay of breast cancer treatment is surgery when the tumor is localized. At present, the treatment recommendations after surgery (adjuvant therapy) change every two years depending on clinical criteria (age, type of cancer, size, metastasis), and patients are roughly divided to high risk and low risk cases *(11)*.

The adjuvant therapy may include hormonal therapy (with tamoxifen or an aromatase inhibitor) *(12)*, chemotherapy, immune therapy, and/or radiotherapy. The presence of estrogen and progesterone receptors in the cancer cell is important in guiding treatment. Those who do not test positive for these specific receptors will not respond to hormone therapy. Likewise, HER2/neu status directs the course of treatment *(13)*. Patients whose cancer cells are positive for HER2/neu have more aggressive disease and may be treated with trastuzumab, a monoclonal antibody that targets this protein.

2.2.1.4 Chemoprevention and Chemotherapy

Due to failure and severe toxicity of cancer chemotherapy, several alternative medicine approaches are increasingly being claimed to be safe and effective. Current

advances in drug development have revealed cancer preventive and curative efficacies of many phytochemicals *(14)*. For these reasons, there has been a global trend toward the use of natural substances present in fruits, vegetables, oilseeds, and herbs as antioxidants and functional foods.

Vegetables and fruits contain several classes of phytochemicals that have antioxidative, antimutagenic and anticarcinogenic effects, making plants useful for treating cancer and other diseases in humans. For examples, some vitamins and their derivatives have important biological roles related to cancer prevention and free radical scavenging. Some phytochemicals, such as Taxol, Oncovin, and captothecin, are spotlighted in current clinical use for cancer treatment.

For a variety of reasons naturally occurring dietary substances over synthetic agents are preferred by patients to prevent cancer. There has been an increasing interest in dietary compounds that have an innate ability to modify deregulated intracellular pathways thereby delaying process of carcinogenesis. While fruits and vegetables are recommended for the prevention of cancers and other diseases, the molecular mechanisms of activities still need to be clarified.

2.2.2 *Solanum nigrum* Linn

2.2.2.1 Traditional fork medicine

Solanum nigrum Linn (SNL) is an herbal plant that commonly grows in the pen fields of temperate climate zones of Asia. It has been used in traditional folk medicine *(15)* and is believed to have various biological activities. For example, Chinese people use it to cure inflammation *(16)* and edema by its antipyretic and diuretic effects. It is used as hepatoprotective agent *(17)* and for treating various kinds of tumors *(18, 19)*, including liver cancer, breast cancer, lung cancer, stomach cancer, colon cancer, and bladder cancer.

2.2.2.2 Scientific research

Recently, some reporters have shown the antioxidant and antitumor activities. The extracts of SN suppressed the oxidant- mediated DNA-sugar damage, exerted cytoprotection against gentamicin-induced toxicity on Vero cells, induced apoptosis in MCF-7 cells *(20)*, induced necrosis in SC-M1 stomach cancer cells, induced autophagy and apoptosis in Hep-G2 liver cancer cells *(21)*, inhibited 12-*O*-tetra-decanoylphorbol-13-acetate-induced tumor promotion in MCF-7 cells *(22)*, and anti-neoplastic activity against Sarcoma 180 in mice. It has also been demonstrated that an ethanol extract from fruits of SNL had a remarkable hepatoprotective effect in CCl_4-induced liver damage *(17)*, and inhibited the proliferation of human MCF-7 breast cancer cells and induced cell death by apoptosis. The SN ethanol extract

suppressed the oxidant mediated degradation of calf thymus DNA. These studies suggest that SN possesses a beneficial activity as an antioxidant and antitumor-promoting agent, although the mechanism for the activity remains to be elucidated.

2.2.2.3 Active phytocompounds

The whole plant of the Solanum nigrum has also been detected to contains many steroidal glycosides, steroidal alkaroids, steroidal oligoglycosides *(18, 23, 24)*, including solamargine, solasonine, solavilline, solasdamine, and solanine. The activities of such alkaloids could be used for anti-tumor purposes. Besides, it has been reported to contain many polyphenolic compounds *(20, 21)*, including gallic acid, protocatechuic acid, catechin, caffeic acid, epicatechin, rutin, naringenin, that the antioxidant and antitumor activities may be due to the presence of polyphenolic constituents. Furthermore, a 150 KDa glycoprotein isolated from *Solanum nigrum* Linne has been reported could reduce inducible nitric oxide (iNO) production, and has an apoptotic effect in HCT-116 cells *(25)*.

2.2.3 Autophagy

2.2.3.1 Cellular homeostasis

Cell homeostasis is maintained by a precisely regulated balance between synthesis and degradation of cellular components. There are 2 powerful hydrolytic mechanisms in eukaryotic cells: the proteasome (ubiquitin dependent protein degradation system) and the lysosome/ vacuole *(26)*. There are at least 3 different pathways for lysosomal protein degradation: Cvt (cytosol to vacuole targeting pathway), Vid (vacuolar import and degradation pathway), and autophagy.

2.2.3.2 Process and target selection

Autophagy or "self-eating" is an evolutionarily conserved catabolic process where cytoplasmic contents and organelles (including long-lived proteins and damaged organelles) are deliverd to the lysosome, where degradative enzymes break the vesicle down *(27)*. Degradation and recycling of the contents of the vesicle enable the cell to continue to carry out essential processes for cell survival under stress conditions.

Autophagy is basically a non-selective process, in which bulk cytoplasm is randomly sequestered into the cytosolic autophagosome *(28)*. However, in some cases it may select its target. For example, autophagy can selectively eliminate some organelles, such as injured or excrescent peroxisomes, endoplasmic reticulum (ER) and mitochondria *(29)*.

2.2.3.3 Three types of autophagy

There are three types of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy, and the term “autophagy” usually indicates macroautophagy unless otherwise specified *(27).*

Macroautophagy comprises the following processes: initially, cytoplasmic components, including proteins and organelles, are engulfed by double-membrane structures known as autophagosomes. Then the autophagosome targets the lysosome, where its outer membrane fuses with the lysosomal membrane and the inter sac (autophagic body) enters the lysosome/vacuole (name autophagolysosome or autolysosome) where the contents are degraded into their components (for example, from proteins to amino acids). After degradation of the cargo, the breakdown products are released into the cytoplasm, where they presumably provide a source of metabolites to support biosynthesis and other processes.

Microautophagy is a form with few features. In this pathway, the membrane of the lysosome invaginates, and then finally pinches off to form an internal vacuolar vesicle that contains material derived from the cytoplasm. The difference between macroautophagy and microautophagy is that in microautophagy the cytoplasm is directly uptaken into the lysosome.

Chaperone-mediated autophagy, differs from the other lysosomal degradation pathways is that vesicular traffic is not involved. Cytosolic proteins with particular peptide sequence motifs are recognized by a complex of molecular chaperones, then bind to a receptor of the lysosomal membrane, the lysosome-associated membrane protein (Lamp) type 2a, proteins are then delivered to lysosomes.

2.2.3.4 Function, survive, and disease

Autophagy has a fundamental role in normal organisms, allowing them to adapt to and survive in variant of stress *(30)*. Recent it has further been found that autophagy may be a transitory tactical response and has a greater variety of physiological and pathophysiological roles *(31)* than expected, such as starvation, adaptation, development, intracellular protein degradation, organelle clearance, anti-aging, elimination of microorganisms *(32)*, cell death, tumor suppression, and antigen presentation.

To understand the various roles of autophagy, it may be simply subclassified into "induced autophagy" and "basal autophagy". The induced is used to produce amino acids following starvation, while the basal is important for constitutive turnover of cytosolic components *(28)*. A great number of extracellular stimuli such as starvation, hypoxia, growth factor deprivation, hormone or chemotherapy as well as intracellular

stimuli like accumulation of misfolded aggressive proteins, ER stress, calcium homeostasis, invasion of microorganisms are able to modulate the autophagic response *(34)*.

Moreover, there is a potential link between autophagy and a number of diseases in humans. For instance, cancer, neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases *(33)*, cardiomyopathy, amyotrophic lateral sclerosis and prion diseases are all associated with increased autophagy activity.

2.2.3.5 Molecular mechanism

The molecular mechanisms *(27)* required for autophagosome formation have been studied by genetic screening of the yeast *Saccharomyces cerevisiae* by the discovery of the *APG* genes. Two ubiquitin-like systems, Apg12 and Apg8, are involved in formation of autophagy. Apg12 can be activated by the E1 enzyme like ATP-dependent protein, Apg7. After the activation, Apg12 is transferred to conjugate with Apg10 then binding with Apg5 and associated with Apg16. The Apg12-Apg5-Apg16 protein complex is essential for autophagy formation.

The other system is Apg8 dependent pathway. Apg8 is modified and activated by protease like protein, Apg4. After modification, Apg8 also required Apg7 to activate and transfer to Agp3. Finally, Apg8 conjugates with phosphatidylethanolamine (PE).

Apg8-PE structure may involve in membrane structure formation in autophagosome. Apg4 is essential for de-conjugating the Apg-PE structure.

LC3 (Microtubule-associated protein 1 light chain 3) *(35)*, the mammalian orthologue of Apg8, targets to the autophagosomal membranes in an Apg5-dependent manner and remains there even after Apg12-Apg5 dissociates. Thus LC3 is currently the only reliable marker of autophagosomes.

2.2.3.6 Regulation

The regulation of autophagy is a very complex process *(36)*. Many signaling pathways play important roles in regulating autophagy. For example, recent studies have shown that formation of autophagy is regulated by the class I and class III phosphatidyl- inositol 3-kinase (PI3-K) signaling pathways *(37)*.

Activation of class I PI3-K mediated by insulin receptor and insulin receptor substrate proteins leads to activate Akt/protein kinase B (PKB). This signal is known to provide survival signal in suppressing apoptosis and autophagy formation. Activated Akt/PKB suppresses the positive regulator of autophagy, the tuberous sclerosis complex (TCS) 1 and 2 proteins that inhibit the small G protein Rheb. Rheb regulates the target of rapamycin (Tor), and Tor inhibits the formation of autophagy. However, the detail mechanism of how Tor kinase inhibits the formation of

autophagy is still unclear.

Activation of class III PI3-K, which includes Beclin 1 in its complex, promotes the nucleation of autophagic vesicles. This complex was found in the *trans*-Golgi network (TGN), suggesting the possibility that autophagy is controlled by providing PtdIns 3-posphate from the TGN to the isolation membranes. Additionally, other factors like TNF, TRAIL (TNF-related apoptosis-inducing ligand), ERK1/2, and ceramide may contribute to the regulation of autophagy.

2.2.3.7 Type II program cell death

Autophagy would involve in maintaining the balance between cell growth and cell death *(34, 38)*. In addition to its role in cell survival, autophagy has been considerd as a non-apoptotic cell death (namely type II programmed cell death) following various stimuli. Some studies suggest excessive autophagy results in type II programmed cell death. However, little is known about the mechanism of autophagy in type II cell death.

Caspase 8 induced-cell death can be shifted to through JNK-beclin 1-mediated autophagic cell death after inhibition of caspase 8 downstream signaling. The function of Bcl-2 family serves as pro-apoptotic or anti-apoptotic signaling molecules. However, cells lacking of BAX and BAK can resist to apoptosis but cells also die

through the autophagic cell death. Tumor-necrosis factor or Fas-Fas ligand signaling pathway can activate caspase independent pathway. The dying cells exhibit the autophagy like morphology. Caspase inhibitor-induced autophagic cell death is severely affected by RNA interference with ATG7 and beclin 1 expression.

2.2.3.8 The discrimination between autophagy and apoptosis

It is still unclear whether autophagy directly induces cell death or it just is the secondary effect of apoptosis. And the distinction between type I and type II PCD is not usually clear. Autophagy can be instrumental in type I cell death-apoptosis, and there are several cases of type II cell death where some traits of apoptosis have been observed. Gonzalez shows that formation of autophagy presents before apoptotic cell death. Which indicate that accumulation of autophagic vacuoles can precede apoptotic cell death, and the inhibition of autophagy triggers type I cell death. Some scientists suggest apoptosis and autophagy are probably evolutionary-related processes.

Expression of Bax, a cell death agonist of Bcl-2 family in Jurkat T-cells is sufficient to trigger apoptosis. However, blocking caspases did not prevent Bax-induced cell death, as autophagic cell death was initiated. Anti-sense down-regulation of Bcl-2 protein in human HL-60 cells induces an autophagic cell death program, which is not reversible by caspase inhibitors and which does not depend on cell death

mitochondrial signaling. Besides, apoptosis and autophagic cell death can also share common inhibitory or activating signaling pathways in mammalian cells as has been demonstrated for Akt/PKB and mTOR.

2.2.3.9 Survival or death

Autophagy can promote survival in stressful or nutrient-deprived conditions. Inhibition of autophagy triggers type I cell death. Furthermore, inhibition of autophagy was shown to increase susceptibility of cells to various stimuli including interleukin-3 (IL-3) deprivation, starvation, and infection.

In contrast, there is considerable controversy regarding the role of autophagy in cell death. There is some pharmacologic and genetic evidence *in vitro* supporting a requirement for autophagy in the cell death response to nerve growth factor (NGF)-deprivation or cytosine arabinoside, serum and potassium deprivation, tumor necrosis factor-α (TNF), caspase-8 inhibition, brevinin-2R, etoposide and staurosporine.

Therefore, autophagy has been associated with both cell death and survival depending on cellular context and stimulus. It is imperative to delineate the effect of autophagy induction and inhibition on cell death in a stimulus-specific manner.

2.2.4 Specific aim

Chow et al. have demonstrated that the water extract of Solanum nigrum Linn could induce apoptosis and autophagy in Hep-G2 liver cancer cells dependent on high or low dose. In our lab, we first get the water extracts of SN leaf, stem, raw fruit (no ripe fruit because sample collection is difficult) for comparing different part on the cytotoxic effect. After comparing the toxicity to liver cancer, breast cancer and relative normal cells, NIH-3T3 fibroblast, SN raw fruit performed more toxicity to NIH-3T3. SN stem performed more toxicity to immortalized HBL-100 breast epithelial cells. SN leaf extracts perform relative low toxicity to NIH-3T3 and that AU565 breast cancer is most sensitive to SN leaf extract unexpected. So here we identified how SN leaf affected AU565 and if SN is suitable for treat AU565 relative breast cancers.

2.3 Material and Methods

2.3.1 Water Extract of *Solanum nigrum* Linn

100g dried *Solanum nigrum* Linn (Leaf, Stem, or Green fruit) were immerse by 2000ml DDW for 30min and boil 95℃ for 30min. Cooled the extracts at room temperature and then filtrated by paper filter. The extracts were dry into powder by Rotavapor and freeze dryer. The dry weight of the extracts was dissolved in DDW and percolated by gel filter (0.22μm).

2.3.2 Cell Lines and Cell Cultures

Human breast cancer lines, like AU565 which over-expresses Her-2, MCF-7 which expresses the basal level of Her-2, and HBL-100 which is derived from a normal human breast tissue transformed by SV40 large T antigen and expresses a basal level of Her-2 were used in this study. All breast cancer cell lines and Hep-G2, NIH-3T3 were purchased from ATCC, and cultured in DMEM supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. These cells were grown at 37℃ in a humidified atmosphere of 5% CO2.

2.3.3 Cell Viability Assays

The effect of SN extracts on cell viability was examined by MTT assay. Briefly,

cells were seeded in a 24-well flat-bottomed plate overnight. Cells were then treated with varying concentrations of drugs. After incubation for various times, 300 μl of MTT solution (0.5 mg/ml, Sigma Chemical Co.) was added to each well and incubated for 4 h at 37 ℃. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in DMSO. Finally, the absorbance was monitored by a microplate reader at a wavelength of 550 nm.

2.3.4 Cell Proliferation Assay by Cell Counting

The trypan blue dye exclusion assay was used to determine the cytotoxic effect of drug on cells. Briefly, cells were seeded on 6-well plases for overnight and then incubated with different concentration of drugs for varying time. At the end of the incubation periods, The cels were harvested by trypsinization. The cells were mixed well with trypan blue solution and the live cells without dye staining inside counted by a hemocytometer under a microscopy.

2.3.5 Western Blot Analyses and Antibodies

Cells were harvested and homogenized by using the Golden lysis buffer (10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris-HCl pH 7.9, 100 μM

β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 30 min at 4℃. Protein content was determined against a standardized control, using the Bio-Rad protein assay kit (Bio-Rad Laboratories). The protein inputs in the western blot analyses were normalized by loading equal amounts of total protein lysates into the SDS-PAGE gel. Transferred onto polyvinylidene difluoride membranes, and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. Reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL). The intensity of the bands was scanned and quantified with National Institute of Health Image software.

Antibodies against Akt, phospho-Akt (Ser^{473}), AMPK, phospho-AMPK (Thr^{172}) and c-PARP were purchased from Cell Signaling Technology (Beverly, MA); Antibodies against LC-3 and phospho-mTor; Anti-β-actin antibody was purchased from Sigma Chemical Co. (St. Louis, MO).Anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA);

2.3.6 ATP Level Determination

Regarded to the protocol of ATP lighting kit. Cells were incubated in 96-well

flat-bottomed plate overnight. Cells were incubated with ATPase light kit after drug treated for varying time. The cells were lysed with lysis buffer, transfered to a light-avoid black 96-well dish, and the luminescence were measured by an ELISA reader.

2.3.7 Flow Cytometric Analysis of Apoptosis, Cell Cycle, ROS, MMP, and the Content of Mitochondria

For the flow cytometric analysis, cells were seeded on 60mm petri-dishes for overnight and then incubated with different concentration of drugs for varying time.

For the analysis of cell cycle and apoptosis, cells were stained with PI (propidium iodide). After drug treatment, cells were trypsinized and washed twice with ice-cold PBS (phosphate-buffered saline), the cells were fixed with 70% ice-cold ethanol overnight at -20℃. Following 1500rpm centrifugation, the cell pellets were treated by RNAase A, stained with PI, and then analyzed by flow cytometry (FACScan, BD Biosciences, Mountain View, CA). The percentage of sub-G1 fraction was analysis by a Cell Quest software (BD Biosciences Mountain View, CA).

Cells were stained with DCFH-DA (2', 7'-dichlorofluorescin diacetate) for the analysis of ROS (reactive oxygen species). After drug treagment, mediums were changed with serum free medium with 10 μM of DCFH-DA. After 30 mins incubation,

cells were trypsinized, washed with PBS, and analyzed by flow cytometry. The shift of the fraction was analysis by a Cell Quest software.

DiOC6 (3, 3'-dihexyloxacarbocyanine iodide) was used for the level of MMP (mitochondria membrane potential), cells were stained with DiOC6 (3, 3'-dihexyloxa-carbocyanine iodide). After drug treagment, mediums were changed with serum free medium with 50 nM of DiOC6. After 30 mins incubation, cells were trypsinized, washed with PBS, and analyzed by flow cytometry. The shift of the fraction was analysis by a Cell Quest software.

For comparing the content of mitochondria in cells, cells were stained with Mitotracker (orange, green, or red) (purchased from Invitrogen). After drug treagment, mediums were changed with new medium containing 100 nM of Mitotracker. After 30 min incubation, cells were trypsinized, washed with PBS, and analyzed by flow cytometry. The shift of the fraction was analysis by a Cell Quest software.

2.3.8 Microscope Observation of Cellular Morphology, Autophagic Vascular Organelle (AVO), Nuclear Morphology, Mitochondria, and Formazan Crystal.

For microscope observation, cells were cultured overnight in 6 well Petri dishes with glass slide inside, and then incubated with different concentration of drugs for varying time. After drug treatment, the cellular morphology was observed directly by light microscope.

In order to observe the AVO (autophagic vascular organelle), cells were stained with serum-free medium containing 10ug/ml of MDC (monodansyl dacarbencine). After 30 min incubation, cells were wash with PBS and fixed with 4% formaldehyde for 30mins. After fixed, cells were washed with PBS and observed by fluorescence microscope.

For Nuclear morphology observation, cells were fixed with 4% formadehyde for 30 mins, washed with PBS, and then incubate with ug/ml DAPI for 30mins. Washed with PBS for 3 times and observed by fluorescence microscope.

For the observed of the content of mitochondria, cells were stained with 100nM of Mitotracker (orange, green, or red; Invitrogen) for 30 min. Washed by PBS and then observed by fluorescence microscope directly.

For the observation of MTT formazan crystal, cells were cultured overnight in 24 well petri dishes without glass slide inside, After MTT reagent incubation, the formazan crystal was observed directly by using a light microscope.

2.3.9 Statistical analysis

All values were expressed as mean ± S.D. Each value is the mean of at least three separate experiments in each group. Student's *t*-test was used for statistical comparison. * indicates the values are significantly different from the control. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

2.4 Results

2.4.1 AU565 was more sensitive to the treatment of the water extract of *Solanum nigrum* Linn leaf

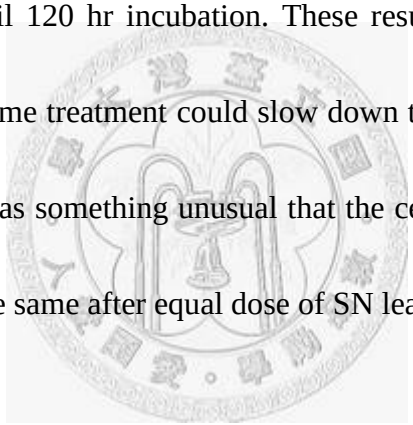
First, we compared the cytotoxic effect of *Solanum nigrum* Linn leaf in different cell lines, including Hep-G2 liver cancer cells, NIH-3T3 fibroblast, HBL-100 immortalized breast epithelial cells, MCF-7 breast cancer cells, and AU-565 Her-2 over-expression breast cancer cells, by MTT cell viability assay. **Figure 1** showed after 10 μg/ml treatment for 24 hr, the cell viability of AU565 decreased to about 55%. In contrast to other cell lines, it was more sensitive to the water extract of *Solanum nigrum* Linn leaf (SN leaf). This suggested that SN leaf may have chemotherapeutic potential in AU565 breast cancer, and for such reason we choose AU565 for further studies.

2.4.2 Below 25 μg/ml of SN leaf treatment, the cell viability and growth rate were decreased, but cell death was not increased.

In order to determine if the cytotoxic effect of SN leaf in AU565 is dose and time dependent. Varying doses of SN leaf (0, 5, 10, 15, 20, 25, 50, 100 μg/ml) were used for different time (6, 12, 18, 24, 48, 72 hr), and the cell viability analyzed by MTT assay was shown in **Figure 2a.** The decrease of cell viability started at the dose of

5ug/ml and at the time of 12hr. When treated with 10 μg/ml of SN leaf, the cell viability decreased down to about 50% at the time of 18 hr. But no more toxic effect was observed after longer time (18-72 hr) or higher doses (10-25 μg/ml) treatment.

To confirm the outcome from MTT cell viability assay, the number of cell death were counted by trypan bule exclusion assay. Accidently the cell death ratios were not different from the control to SN leaf treated groups. **Figure 2b** showed the growth curve but not the cell death number, the doses of SN leaf below 25 μg/ml did not affect the growth rate until 120 hr incubation. These results indicated that SN leaf below 25 μg/ml for long time treatment could slow down the cell growth rate but not induce cell death. There was something unusual that the cell viability was decreased, but the cell number was the same after equal dose of SN leaf treatment.



2.4.3 SN leaf extract decreased the formazan crystal content in AU565 cells

It is queer that after treating with 10 to 25 μg/ml of SN leaf for 24hr, the cell viability analyzed by MTT assay was decreased, but the cell number not. For such reason, we particularly observed the MTT formazan crystal by light microscope. As shown in **figure 3**, it is interesting that the formazan crystal content decreased evidently, and such phenomenon is constant. There may be two reasons that cause of the decreasing formazan crystal in AU565 cells, the first one is the mitochondria

eliminated by autophagy after SN leaf treated. And the second one, the dehydrogenase which transfers tetrazoliumin in MTT reagent to formazan was inhibited after SN leaf treatment.

2.4.4 SN leaf decrease the ATP level of AU565 cells

If mitochondria was dysfunctional or eliminated after SN leaf treatment, the ATP level would reduce because the energy factory was diminished. Here, we measured the ATP level by ATPase lighting kit. In **figure 4**, the ATP content slowly reduced down to about 60% when compared to control. This result suggested that SN leaf treatment may affect mitochondria then decreased the ATP level in cells. But still other possibilities may contribute such effect.

2.4.5 The AVO formation increased after SN leaf treatment.

In order to know whether the SN leaf treatment will induce autophagy, cells were stained with MDC to detect the AVO (Autophagic Vacuole Organisms) formation. **Figure 5a-d** showed the punctuated MDC positive cells at 25 μg/ml of SN leaf treatment as compared to control. **Figure 5e** showed when treated with 25 and 100 μg/ml of SN leaf, the AVO formation increased up to about 45% at the time of 12 hr with a great variation in independent experiments. The AVO positive cells decreased

after 18 hr treatment. These indicated that SN leaf did induce autophagy in AU565 cells, but such induced autophagy was not always kept at a high level.

2.4.6 Below 25 μg/ml of SN leaf treatment, the level of LC-3 in AU565 cells increased before 12 hr, but decreased after that, accompanied with the raising of phospho-Akt

We next tested the protein level of LC3-I (inactivated form) and LC3-II (cleavage form; active form), the protein marker of autophagy. **Figure 6** showed that after 25 μg/ml of SN leaf treatment, the LC-3 level increased with a peak at 6 to 12 hr then decreased after 12 hr. The level of p-Akt and p-mTOR (autophagy and apoptosis negative regulator) did not change before 12 hr, but increased significantly after 18hr treated with SN leaf.

Figure 7a showed that at the time of 12hr treatment the LC-3 level increased dose dependently, but other proteins, including p-Akt and p-AMPK did not change significantly. At 24 hr, showed in **figure 7b**, the LC-3 level did not change after 0 to 25 μg/ml of SN leaf treatment, and the level of p-Akt and p-mTOR increased dose dependent. No matter time or dose test, the p-AMPK, one of autophagy positive regulator, did not change significantly. These results suggested the SN leaf induced autophagy in AU565 cells, but the mechanism seemed not related to the down

regulated of p-Akt or the up regulated of p-AMPK.

2.4.7 SN leaf induced autophagy in AU565 cells reduced the amount of mitochondria slightly.

Whether the SN leaf induced autophagy reduced the level of mitochondria in AU565 cells was tested by staining mitochondria with Mitotrackers. As shown is **Figure 8a, b**, the mitochondria level in cells seemed not significant different between SN leaf treated (25, 50, 100 μg/ml) and control groups. This result suggested that SN leaf induced autophagy was not major to eliminate mitochondria in AU565 cells, and the decreased level of mitochondria was not related to the decrease of formazan crystal (40%).

2.4.8 SN leaf did not induce AU565 cells into cycle arrest or apoptosis below 25ug/ml

To identify with weather SN leaf induced AU565 into cycle arrest or apoptotic cell death. First, DAPI were used to stain nuclear and observed the nuclear morphology because apoptosis could be characterized by DNA fragmentation and chromatin condensation. Compared with control group, **Figure 9a-d** showed no significant difference in the nuclear morphology observation in SN leaf treated group.

Second, as shown in **Figure 9e-f**, the sub-G1, G1, or G2 phase observed by PI staining and flow cytometer were not significant difference between control and SN leaf treated groups. Diosgenin, one alkaloid, has been known to induce apoptosis in AU565 cells was the positive control for the chromatin condensation observation. Third, **Figure 12a-d** showed no more apoptotic bodies were observed when compared with control group. Forth, as our data shown in **Figure 7**, the apoptosis related protein, c-PARP, did not changed. These results indicated that below 25 μg/ml of SN leaf treatment did not induce apoptosis in AU565 cells.

2.4.9 Higher dose, 100ug/ml of SN leaf treatment induced cell death by autophagy or/and apoptotic mechanism.

Before, the studies were focused on the dose below 25 μg/ml that would induce autophagy but not death, here showed the response of AU565 cells after higher dose of SN leaf treatment (50, 100 μg/ml). Compared with 25 μg/ml, the cell viability analyzed by MTT assay reduced more after 50 and 100 μg/ml treated for more than 48 hr **(Figure 10a**). And as showed in **Figure 10b-c**, the cells stop growth (50 μg/ml) and about 20% dead (100 μg/ml) after SN leaf treatment. After 100 μg/ml of SN leaf treatment for 48hr the protein level of LC-3 was more, the level of p-Akt decreased, and c-PARP increased slightly (**Figure 11**). After 100 μg/ml of SN leaf treat, the cells

morphology (**Figure 12g, h**) changed, the corpse looked like withered and with a bit more of apoptotic bodies. The results above indicated that longtime treated with 100 μg/ml of SN leaf could induce cell death by autophagic and/ or apoptotic mechanism.

2.5. Disscussion

2.5.1 The Cell Viability and Cell Death

Solanum nigrum Linn has been reported to have many anti-cancer effect, including apoptosis in MCF-7 breast cancers and Hep-G2 liver cancer cells, necrosis in SC-M1 stomach cancer cells, and autophagy in Hep-G2 cells. In our studies, we first compared different part of SN, and identify that SN leaf was more toxic to AU565 breast cancer cells and moderate to NIH-3T3 fibroblasts. Other cells lines like Hep-G2, MCF-7, HBL-100 had similar viability between AU565 and NIH-3T3 after the treatment with SN leaf.

The cell viability of AU565 attached to about 50% by 10 μg/ml SN leaf treated for 18 hr, but no more decreasing cell viability was observed in the increasing dose to 25 μg/ml. The cells did not die below 25 μg/ml treatment, even the cell growth rate seems reduced after long time treatment. At 100 ug/ml treatment, the cell viability further decreased to about 30%, and the cells were dead (20%) with an autophagic like morphology after 4 days treatment.

2.5.2 The roles of p-Akt

Before, Chow et al showed that the SN treatment induced the down regulation of p-Akt contributed the SN treatment induced autophagy in Hep-G2 cells. In our studies,

100 μg/ml of SN leaf treatment had similar results that the reduced level of p-Akt accompanied with the increasing level of LC-3.

But the doses below 25 μg/ml, the observation that the ATP level decreased, autophagic vacuole organisms increased, the level of LC-3 protein increased, and the less reduced level of mitochondria in cells all indicated that SN leaf induced autophagy in AU565 cells. Studies before shown that autophagy may due to the up regulation of p-AMPK or the down regulation of p-Akt. But in our studies here, the level of p-AMPK was not significant difference and the level of p-Akt did not decrease. The results indicated that the lower dose (25 μg/ml) of SN leaf induced autophagy seemed not through Akt or AMPK pathways. Unfortunately, the molecular mechanisms of lower dose of SN leaf induced autophagy in AU565 cells were not evaluated here.

Autophagy were induced by 25 μg/ml of SN leaf treatment in AU565 cells, but the induced autophagy decreased and accompanied with the increased p-Akt after 12 hr of treatment. The increasing level of p-Akt, which inhibits apoptosis and autophagy, may prevent AU565 cells death. The causes of the raising of p-Akt level were still unknown in this study. There may be some compounds in SN leaf that promoted AU565 cell survive, or that was the cell adapt-response to stress after SN leaf treatment. The fates of AU565 cells would be controlled by p-Akt after SN leaf

treatment. Even higher dose of SN leaf induced AU565 cell death, but the survival signal (p-Akt) raised after low dose of SN leaf treatment suggested it was unsuitable for the treat of AU565 cancer cells.

2.5.3 Autophay, Mitochondria, and MTT Formazan Crystal

Mitochondria could be eliminated by autophagy mechanism, but autophagy doesn't mean that mitochondria must be clean out. Our data suggest SN leaf treating induced autotophagy were not major to clean mitochondria in AU565 cells. Two reasons may cause the decrease of MTT formazan crystal, the dehydrogenase in mitochondria dysfunction or the mitochondria deprivation. The slight elimination of mitochondria by SN leaf seemed not sufficient to contribute to the decreased MTT formazan crystal. The data suggested that MTT formazan crystal decrease was not related to autophagy induced by SN leaf, and something in SN leaf may inhibit the dehydrogenase in mitochondria. Actually, the culture medium became more acid (data not shown), and the ATP level decreased after treatment with SN leaf for one day. We presumed that SN leaf treatment may inhibit mitochondria function and/or promote glycolysis pathway, even there were no evidence to improve these theory.

2.5.4 ATP Level

The ATP level decrease was observed after SN leaf treatment. This decreasing would not due to the reduced cell number because at 25 μg/ml of treatment cell did not die anymore. As we knew, the dysfunction or elimination of mitochondria could induce the ATP deprivation, and ATP deprivation may induce autophagy through AMPK pathway. In our studies, the slowly ATP deprivation was not accompany with the raising of p-AMPK that we suggested the ATP deprivation was not the cause of autophagy but probably the effect of mitochondria dysfunction.

2.5.5 Variation in the Studies

The great variation between our data may due to two reasons, the cell growth condition and the drug quality. After cell seeding, the cells in our experiment were not starved because starvation for 2 hr caused autophagy. That may a reason for the big variation. The other maybe the autophagy inducer (s) in SN leaf was (were) easy oxidized that SN leaf should be prepared more freshly.

2.5.6 Which phytocompound(s) induced autophagy

The exact autophagy inducer in SN leaf is still unknow. Some known pure compounds that may exist in SN leaf, including coumaric acid, ferulic acid, vanillic acid, cinnamic acid, protocatechuic acid, caffeic acid, syringic acid, chlorogenic acid,

gallic acid, naringenin, rutin, catechin, α-chaconine, solanine, diosgenin, were chosen to test their effect in AU565 cells (Figure 4 in appendix). Unfortunately, cell death with the apoptotic body corpses was induced by gallic acid, α-chaconine, solanine, and diosgenin. The SN leaf induced autophagy may not simply due to one compound effect, and it may be a synergistic effect of compound cooperation.

2.5.7 Summary

The treatment of SN leaf induced autophagy in AU565 cells, even the mechanism was still unknown. As showed in **Figure 13**, (a) This induced autophagy (before 12hr) was inhibited by the raising of p-Akt (after 12hr), but high dose (100ug/ml) of SN leaf treatment blocked the raising of p-Akt. (b) SN leaf induced autophagy seemed not to eliminate mitochondria majorly. (c) The ATP deprivation and decreased MTT formazan may due to autophagy induced mitochondria depriviation, the dehydrogenase be inhibited, or the mitochondria dysfunction.

2.6 References

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2.7 Figures

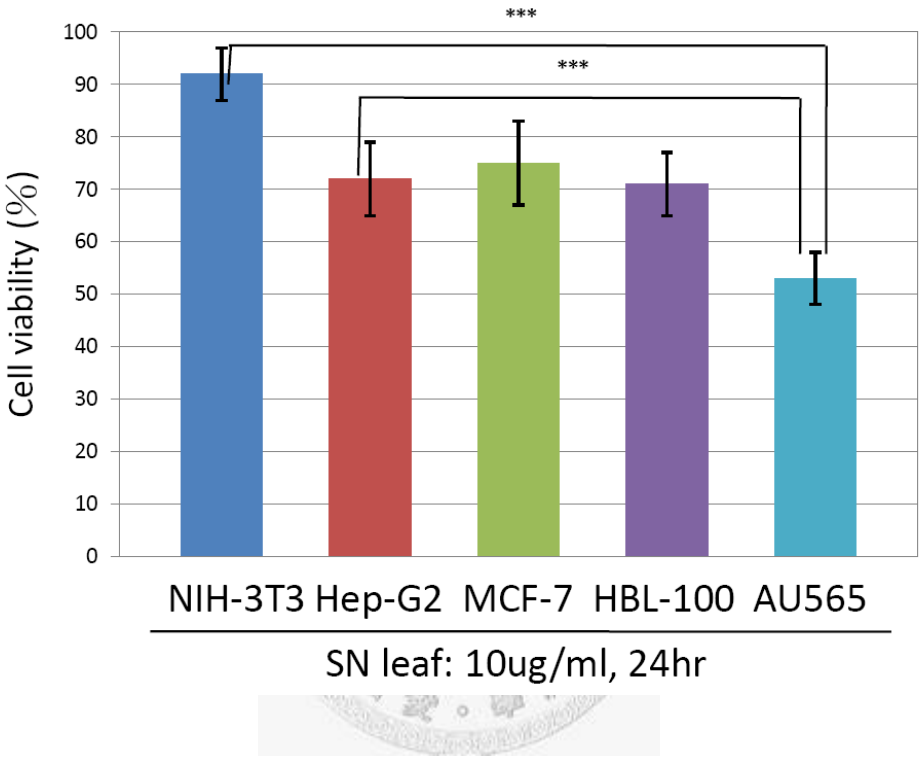
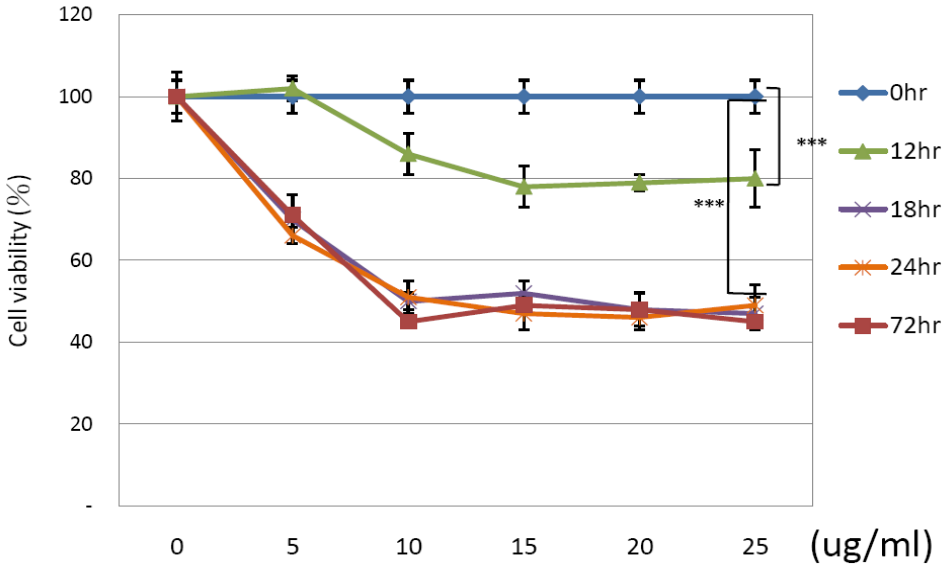


Figure.1. The cell viability comparison of different cells lines treated by SN leaf. MTT assay showed the different response of cells lines treated by 10ug/ml of SN leaf for 24hr. AU565 were more sensitive to SN leaf treatment than HBL-100, MCF-7, Hep-G2, and than NIH-3T3. All values were expressed as mean ± S.D. * indicates the values are significantly different from the control. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), n≧3

a.



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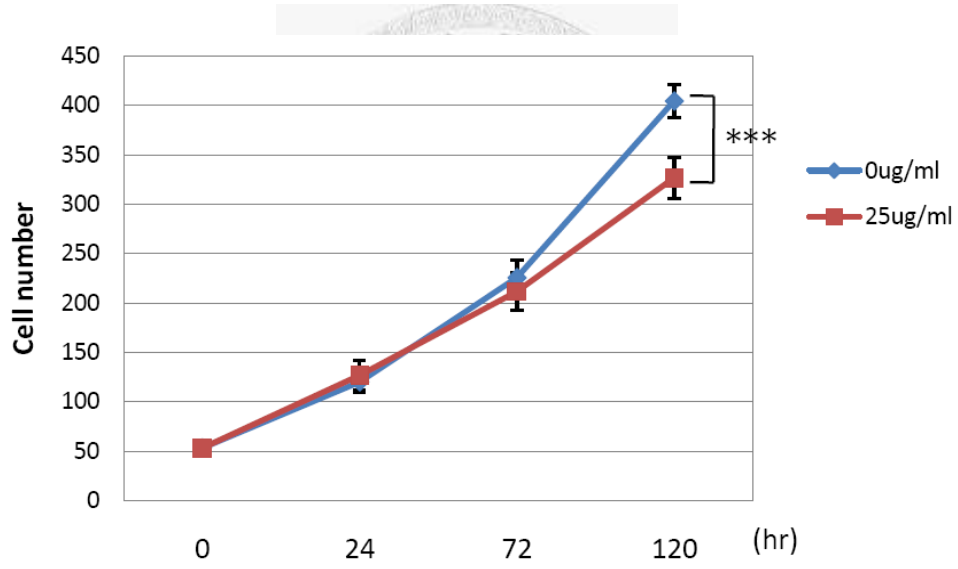


Figure 2. SN leaf treatment reduced AU565 cell viability and the growth rate, but not induce cell death below 25ug/ml.

AU565 were treated by 0, 5, 10, 15, 20, 25 μg/ml of SN leaf from 0 to 120 hr. a. The MTT assay showed the most reduced cell viability to about 50% at 10 μg/ml for 18 hr. b. The trypan blue exclusion assay showed no significant difference on the cell death. Here show that the growth rate reduced after 120 hr treatment of 25 μg/ml SN leaf. All values were expressed as mean ± S.D. * indicates the values are significantly different from the control. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), n≧3

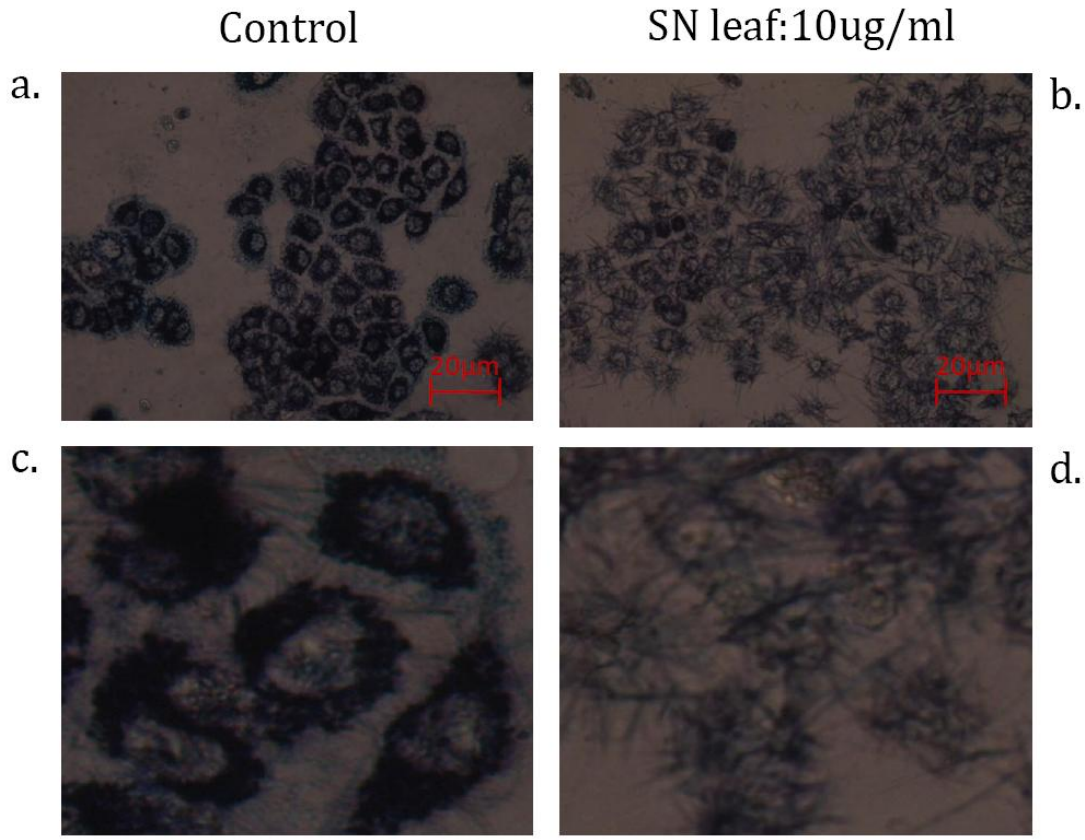


Figure 3. SN leaf treatment reduced the formazan crystal of MTT assay in AU565 breast cancer cells.

SN leaf was used to treat AU565 for 18 hr. The formazan crystal of MTT assay were observed by light- microscopy. After SN leaf treated for 18hr, the formazan crystal decreased obviously. a. and c. 0 μg/ml as control; b. and d. 10 μg/ml. a. and b. were 400 x of original magnification. c. were the field enlarged from a., and d. were the field enlarged from b..

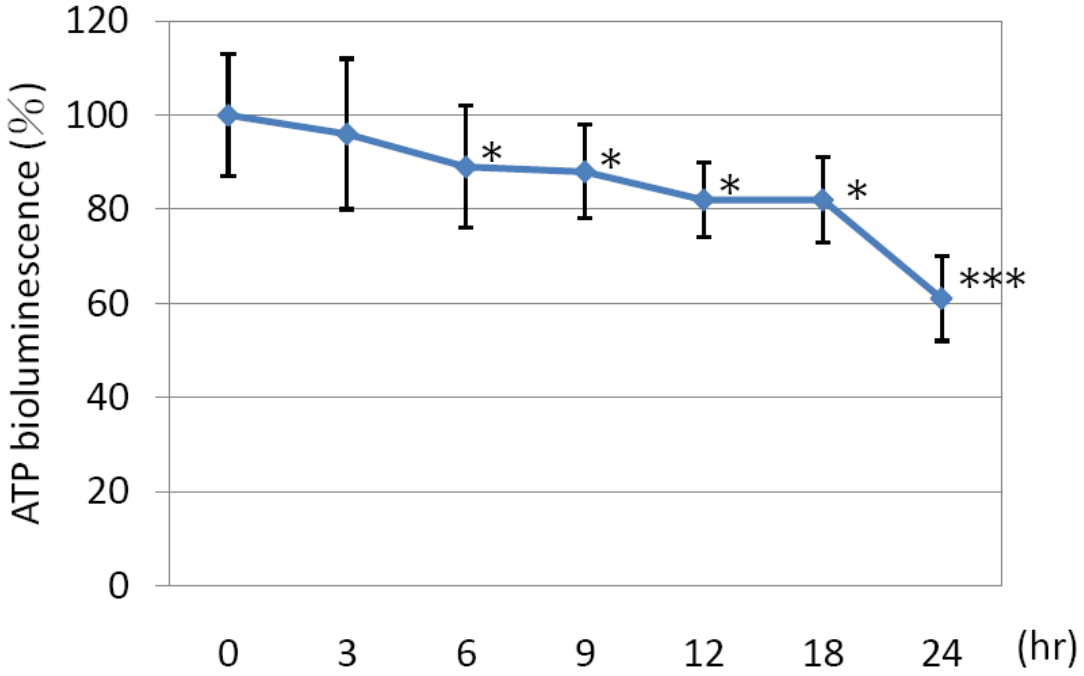


Figure 4. ATP level reduced in AU 565 after SN leaf treatment.

AU565 were treated with 25 μg/ml of SN leaf. The ATP level decreased slowly to about 80% before 18hr. At 24 hr, the ATP content was down to about 60%. The percentage was compared to 0 μg/ml treated control. All values were expressed as mean ± S.D. * indicates the values are significantly different from the control. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), n≧3.

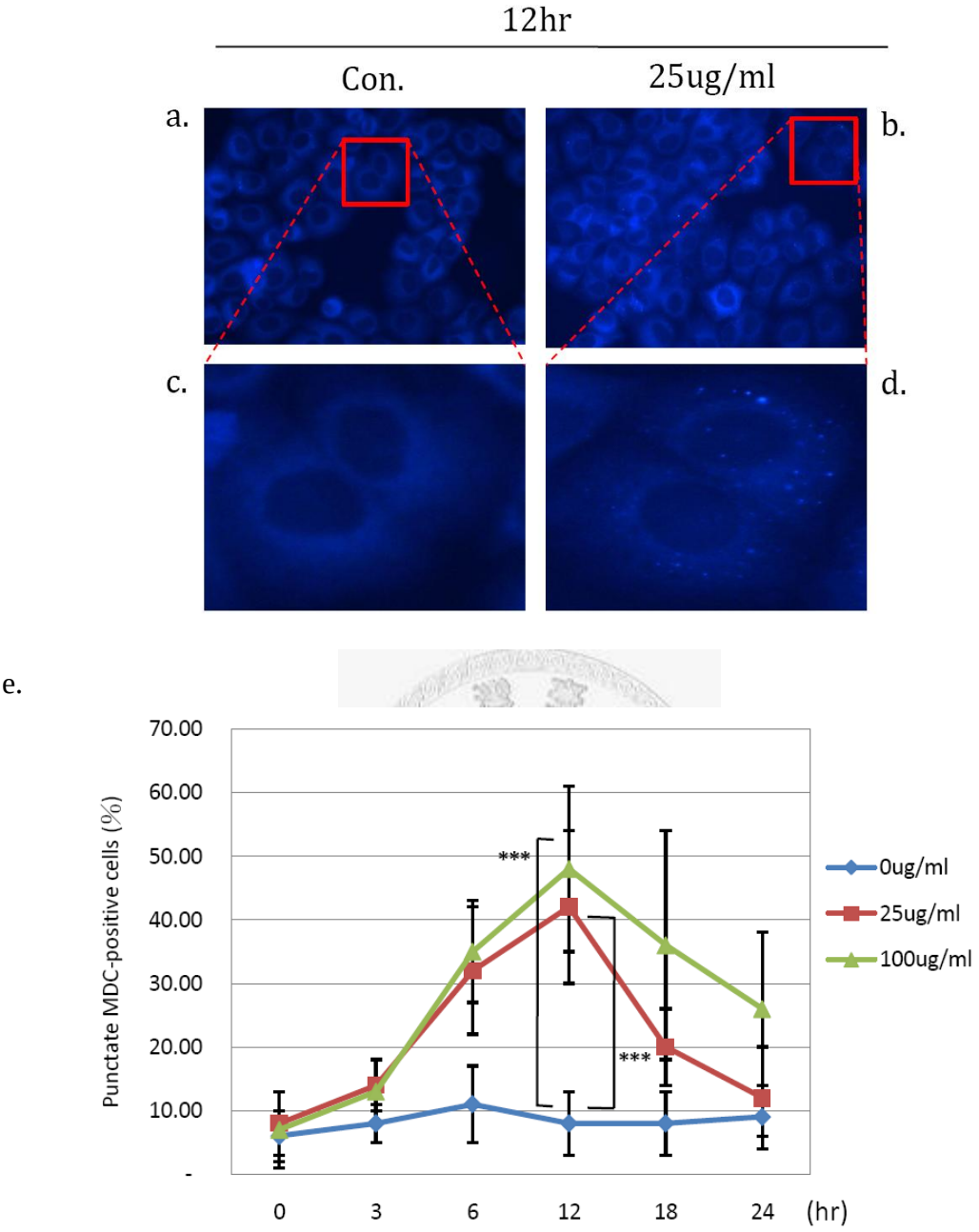


Figure 5. SN leaf treatment induced autophagic vascular organelle (AVO) formation between 6 to 18 hr.

After treating variant concentration of SN leaf for different time. Cells were stained with MDC, the MDC punctuated MDC positive cells increased after 6hr and decrease after 18 hr treatment. a. and c. 0 μg/ml as control; b. and d. 25 μg/ml. a. and b. were 400 x of original magnification. c. were the field enlarged from a., and d. were the field enlared from b.. e. showed the quantification of MDC punctuated MDC positive cells after 0, 25, and 100 μg/ml of SN leaf treatment. n≧3.

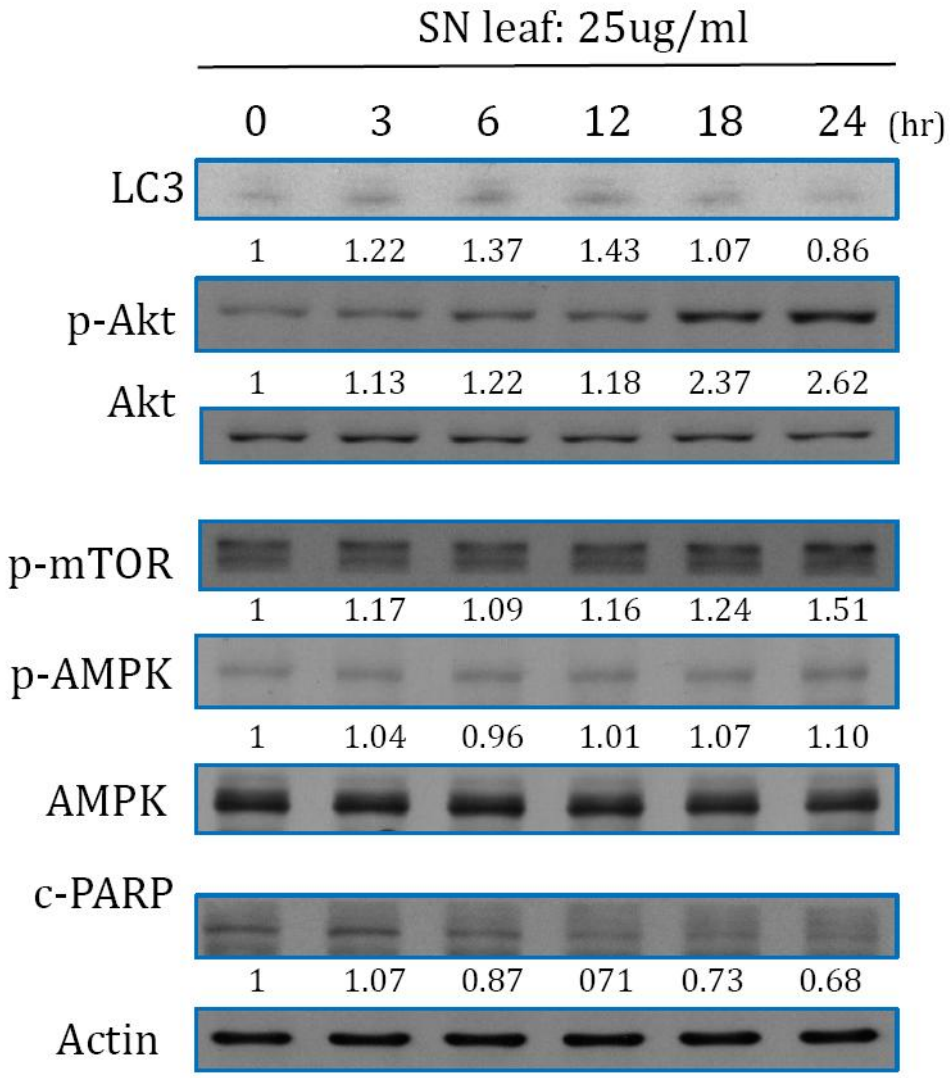


Figure 6. At 25 μg/ml of SN leaf treatment, the level of autophagic marker LC3 increased, but later the apoptosis and autophay repressor, p-Akt, increased.

The result of western blotting showed, 25 μg/ml of SN leaf treatment increased LC3-I, II level begin at 3 hr to 12 hr, and decreased after 18 hr. p-Akt and p-mTOR increased after 18 hr treatment. The level of p-AMPK would not change significantly. Cleavage-PARP decreased time dependent. Immunoblotting with β-actin antibody demonstrated equivalent protein in each lane.

a. b.

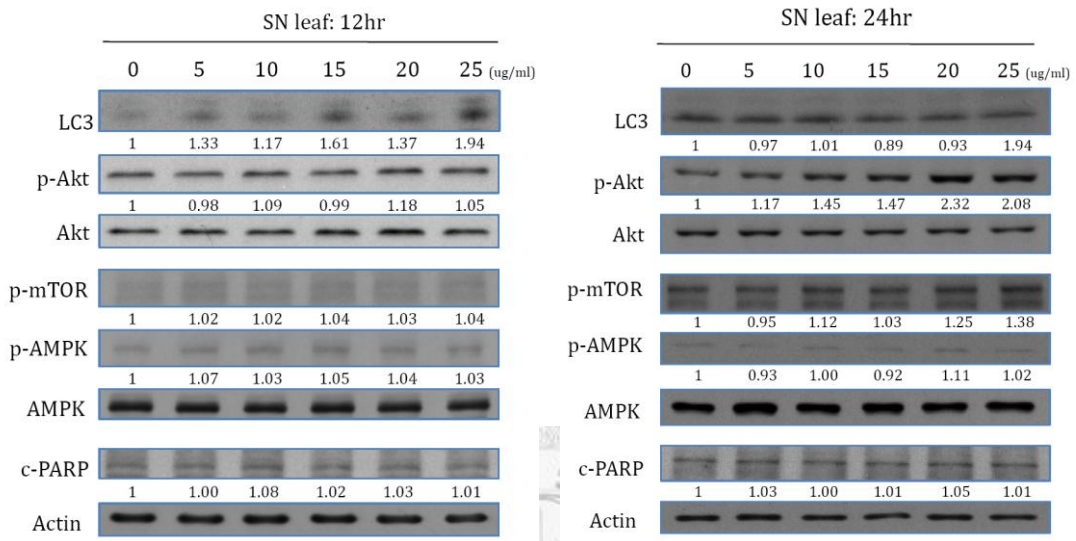


Figure 7. The LC-3 level increased dose dependent at 12 hr. At 24 hr the LC3 without difference between variant concentration below 25 μg/ml, and the p-Akt increased dose dependent.

The result of western blotting showed, a. After 12 hr of different dose of SN leaf treatment, the LC3-I, II level increased dose dependent. The level of other proteins did not change. b. At 24 hr, LC3-I, II were not difference between SN treated and control. The phosphorylation of Akt and mTOR increased dose dependent. The level of p-AMPK and cleavage-PARP would not change significantly. Immunoblotting with β-actin antibody demonstrated equivalent protein in each lane.

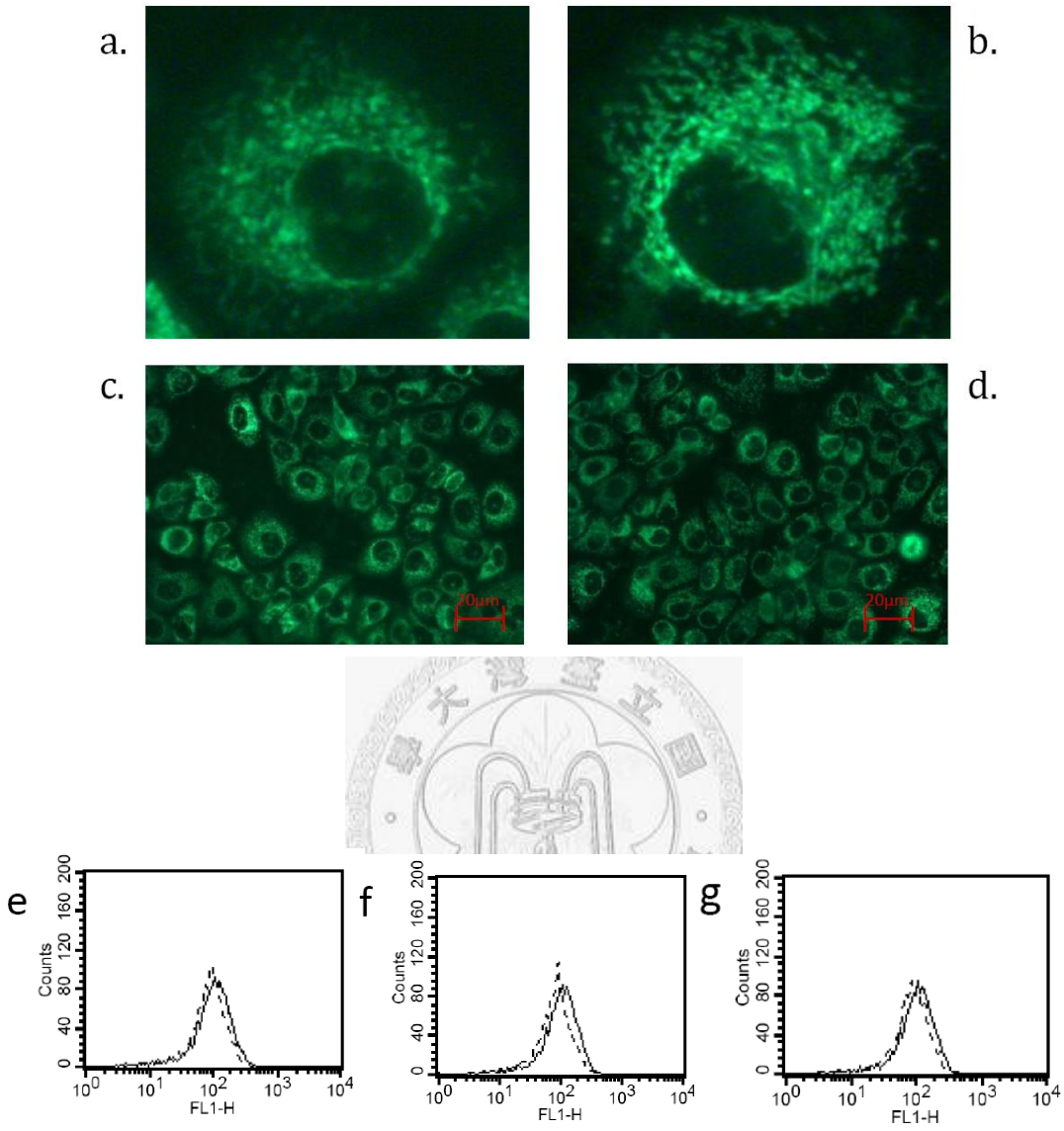


Figure 8. SN leaf treatment did not affect the level of mitochondria in AU565 significantly.

a, b, c, d were the fluorescence observation of cells stained with mitotracker. a, c were the control; b, d were cells treated by 100 μg/ml of SN leaf for 48hr. c, d were 400 x of original magnification and a, c were the field enlarged from c, d. e (25 μg/ml), f (50 μg/ml), g (100 μg/ml) were flowcytometer analysis for comparing the relative amounts of mitochondria in cells. ---; cells were treated with SN leaf, ─; as control, cells were not treated with SN leaf.

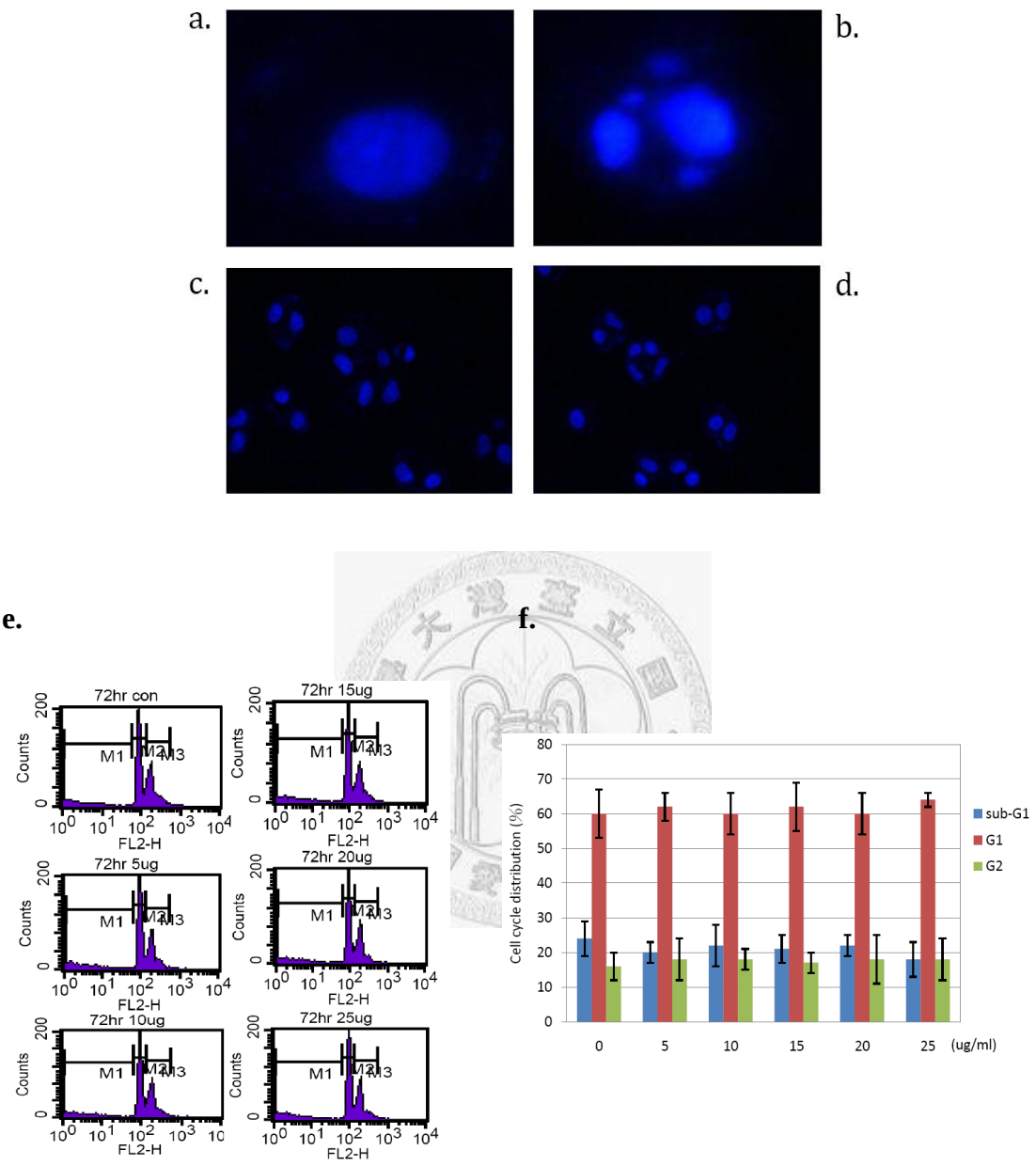
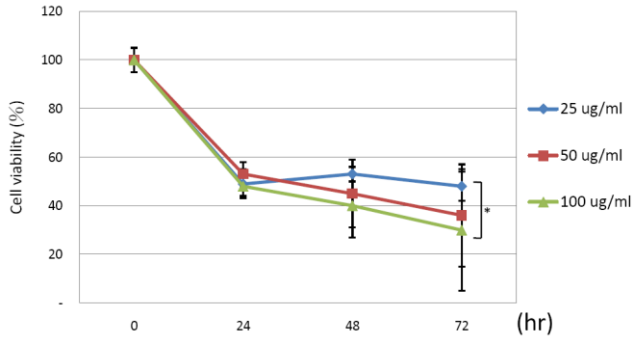


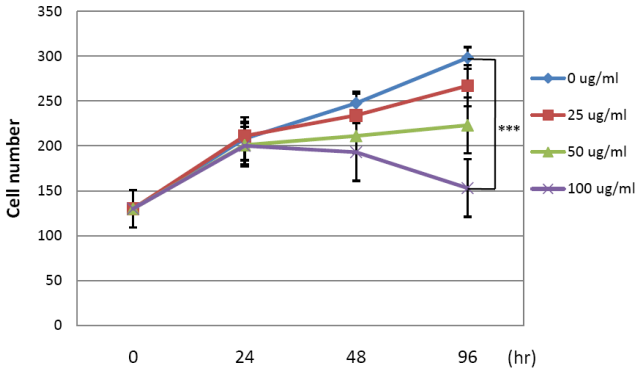
Figure 9. SN leaf treatment below 25 μg/ml would not induce apoptosis, analyzed by DAPI staing and flocytometer.

a. to d. were the nuclear of AU565 stained by DAPI and observed by fluorescence microscope. a. normal nuclear; b. the DNA fragmentation of apoptotic nuclear. c. and d were the nuclear observation of AU565 treated by 0 and 25 μg/ml SN leaf for 48 hr (400 x of original magnification). e. is flowcytometer analysis of AU565 treated by 0 to 25 μg/ml of SN leaf for 72 hr, f. is the quantification of e.

a.



b.



c.

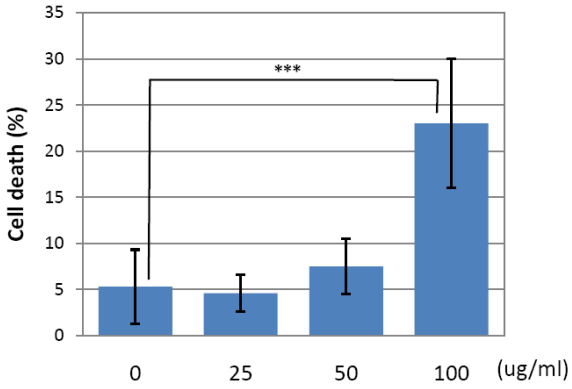


Figure 10. 50 and 100 μg/ml of SN leaf treated for long time could induce AU565 cell death sometimes.

a. MTT assay showed 50 and 100 μg/ml SN leaf could further reduce the cell viability significantly after 72 hr. b, c. Trypan blue exclusion assay showed 50 and 100 μg/ml of SN leaf treatment differ from 25 μg/ml treatment could induce cell death after 48 hr. n≧3

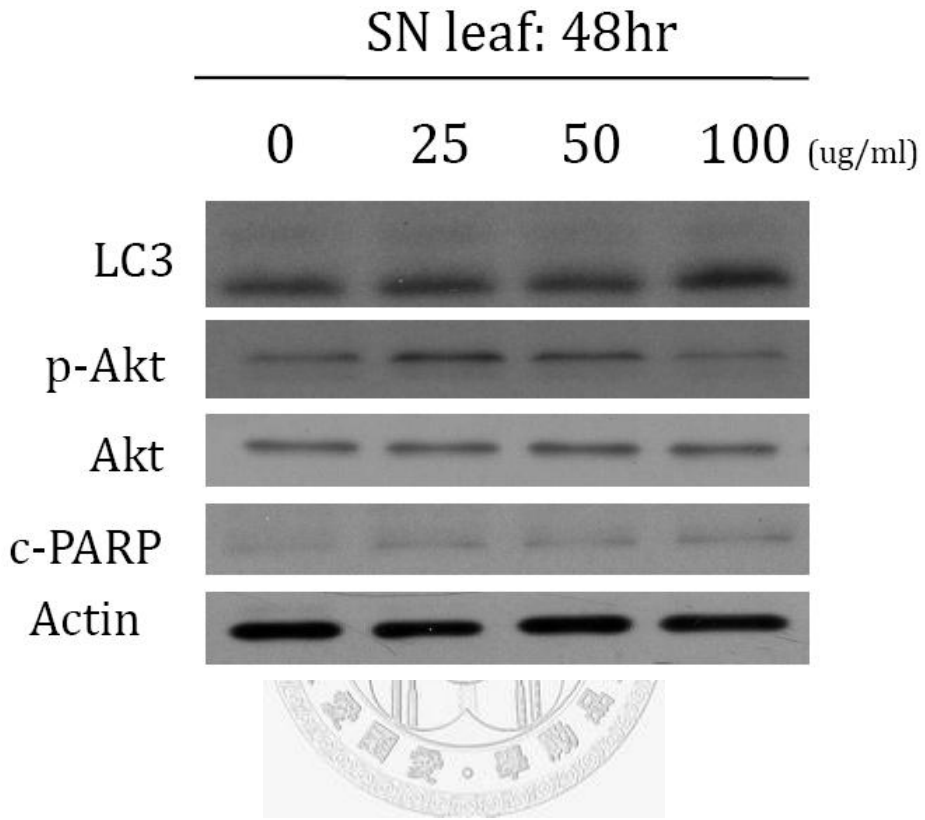


Figure 11. 100 μg/ml of SN leaf treatment may not only induced autophagy but also apoptosis.

After 48 hr of SN leaf treatment, autophagic marker LC3-I, II and apoptotic marker c-PARP increased lightly at 100 μg/ml accompanied with the decreased of p-Akt. Immunoblotting with β-actin antibody demonstrated equivalent protein in each lane.

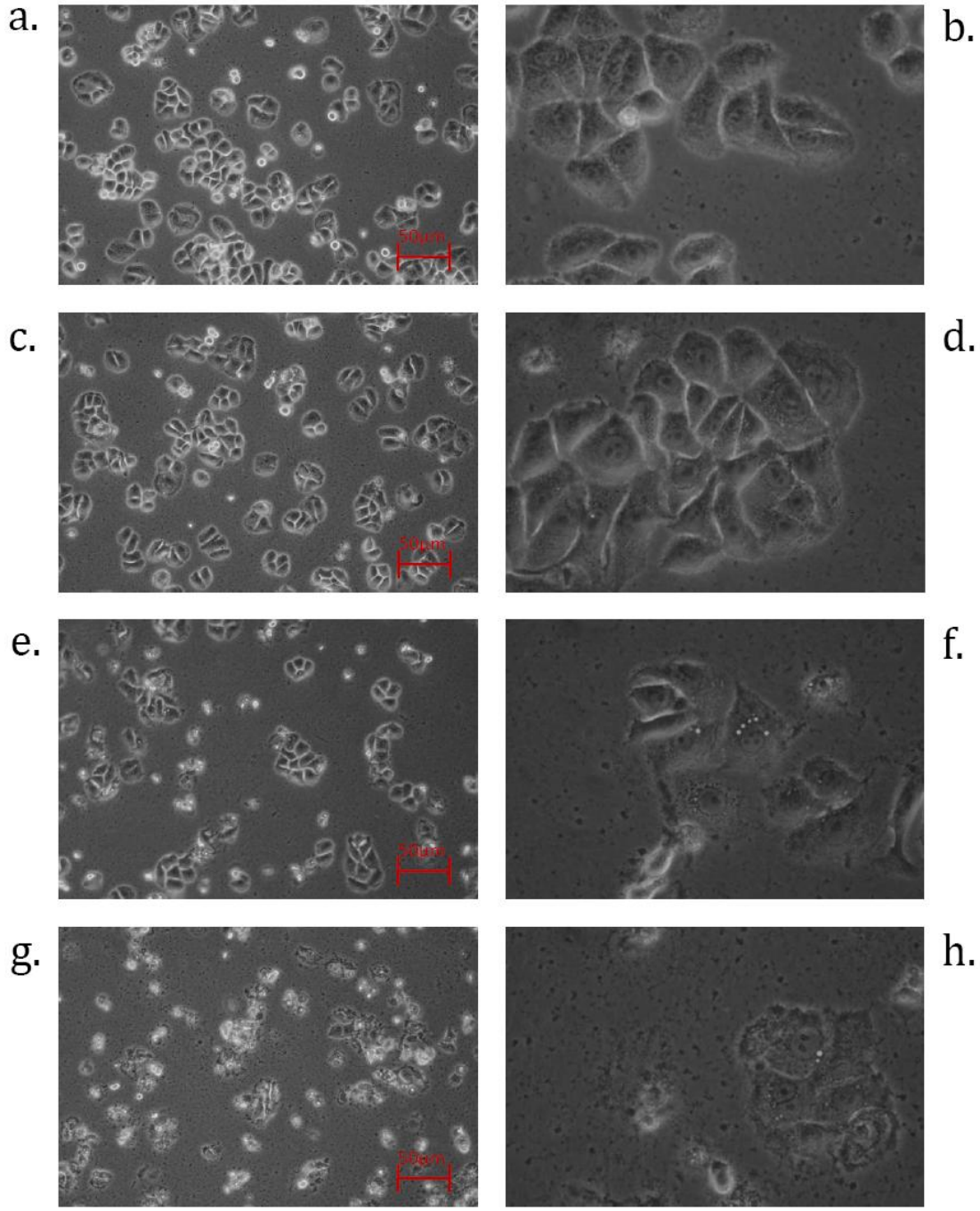


Figure 12. The light microscope observation of AU565 treated by SN leaf.

SN leaf was used to treat AU565 for 72 hr. The cells were observed by light-microscopy. At 50 and 100 μg/ml of treatment, the cell morphology changed and the corpse looked like withered with a bit of apoptotic bodies. a and b 0 μg/ml; c and d 25 μg/ml; e and f 50 μg/ml; g and h 100 μg/ml. a, c, e, g were 100 x of original magnification and b, d, f, h were 1000 x of original magnification.

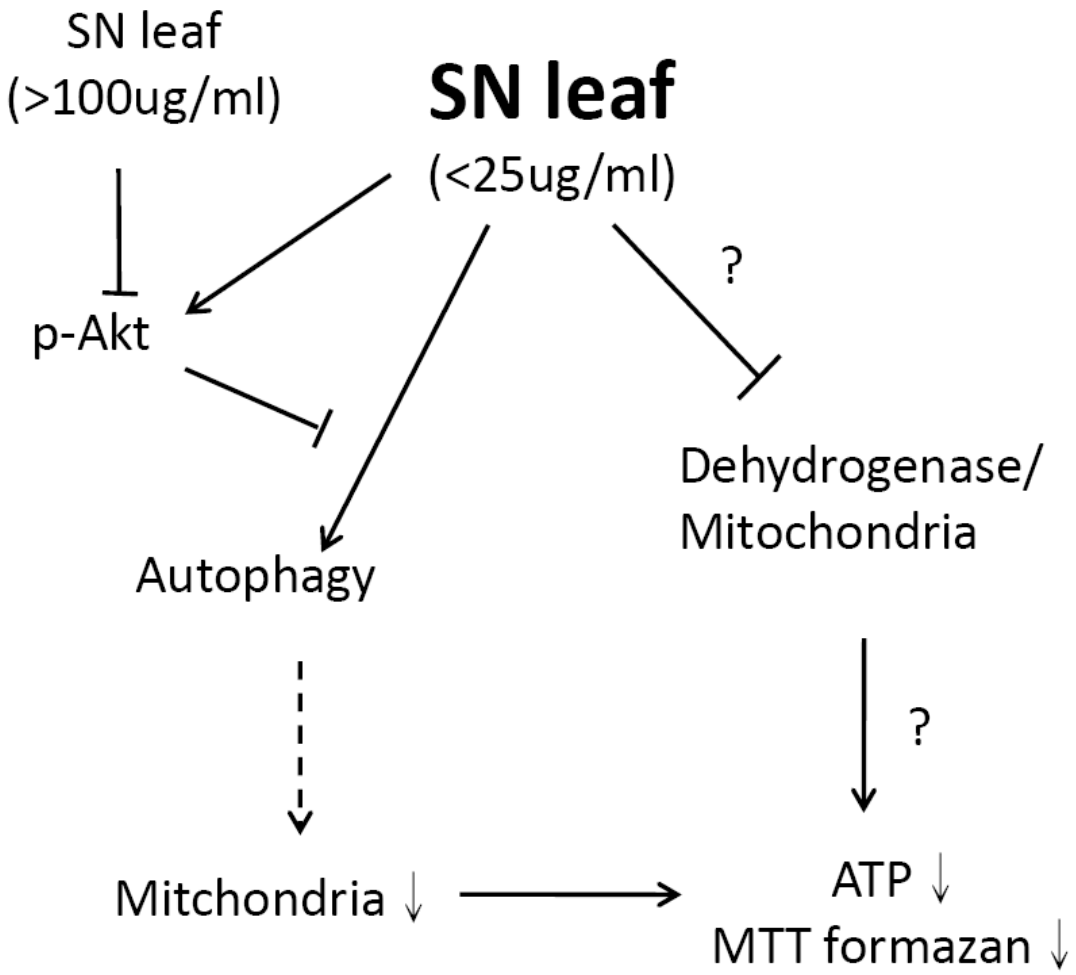
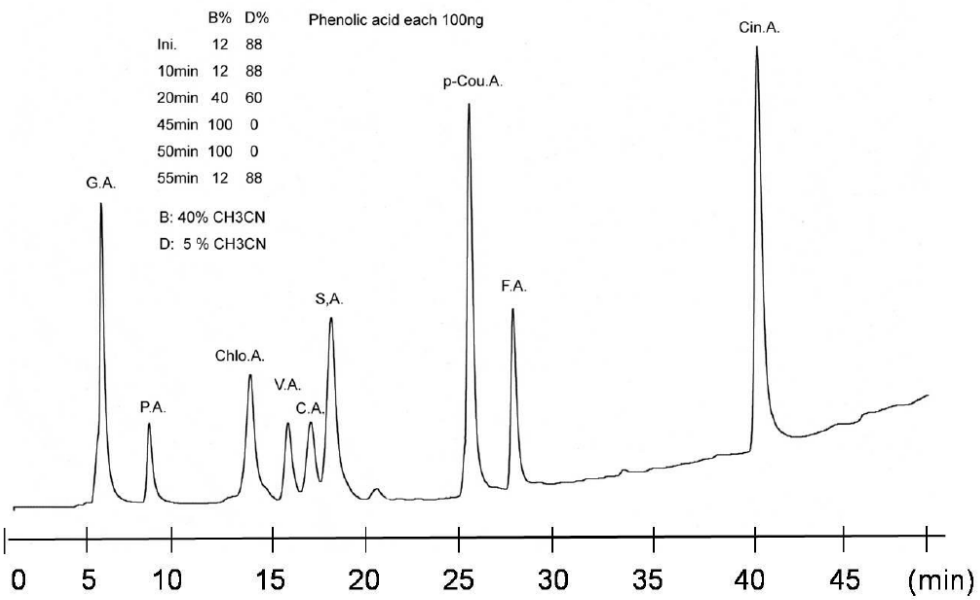


Figure 13. Summary

25 μg/ml of SN leaf induced autophagy in AU565, but such induced autophagy was inhibited by later increased p-Akt. 100 μg/ml of SN leaf inhibited the increasing p-Akt that made cell death through autophagy and apoptosis pathway. SN leaf induced autophagy decreased the level of mitochondria that may reduce the level of ATP and MTT formazan. If the dehydrogenase in mitochondria was inhibited by SN leaf still need to be evaluate

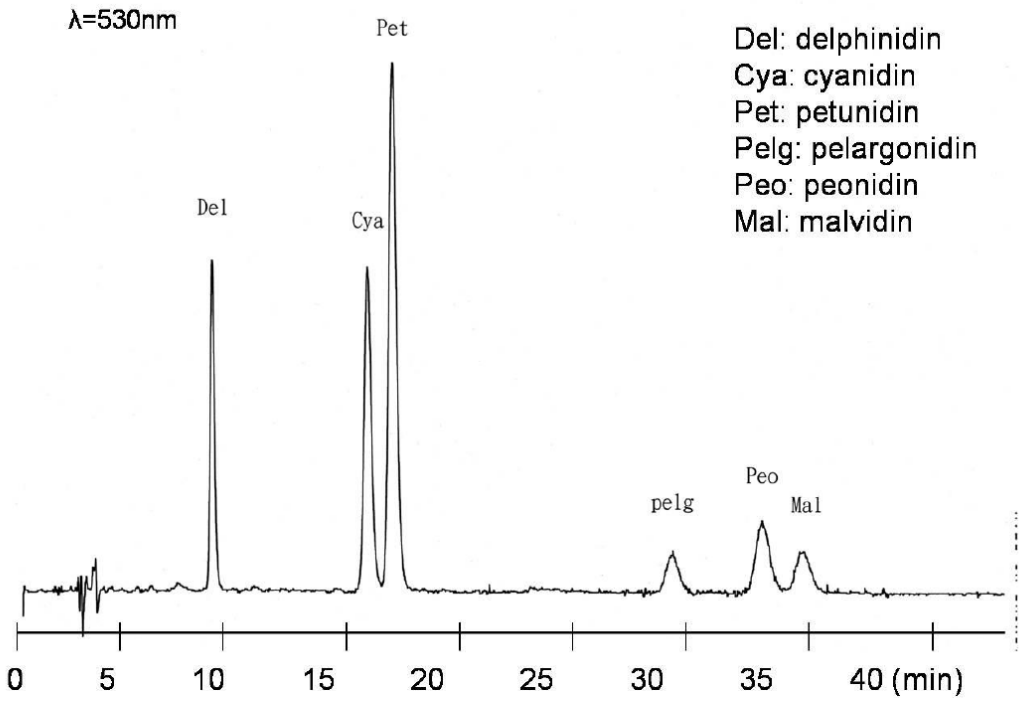
2.8 Appendix



ug/g	G.A.	P.A.	Chlo.A.	V.A.	C.A.	S.A.	p-cou.A.	F.A.	Cin.A
Leaves	11.57	59.05	181.84	7.67	42.05	7.65	718.66	26.59	10.14
Stem	10.76	11.32	87.44	7.81	8.73	0.97	11.36	10.78	1.01
Purple fruits	10.96	21.05	1128.96	0.25	10.14	9.77	85.74	40.49	N.D.
Green fruits	15.47	147.28	2937.94	N.D.	N.D.	13.54	220.95	14.51	N.D.

Appendix 1. Analysis of phenolic acid content in SN extract.

Phenolic acids (chlorogenic acid, coumaric acid, ferulic acid, vanillic acid, syringic acid, cinnamic acid, protocatechuic acid, gallic acid, and caffeic acid) in SN extract were quantitatively analyzed by HPLC.



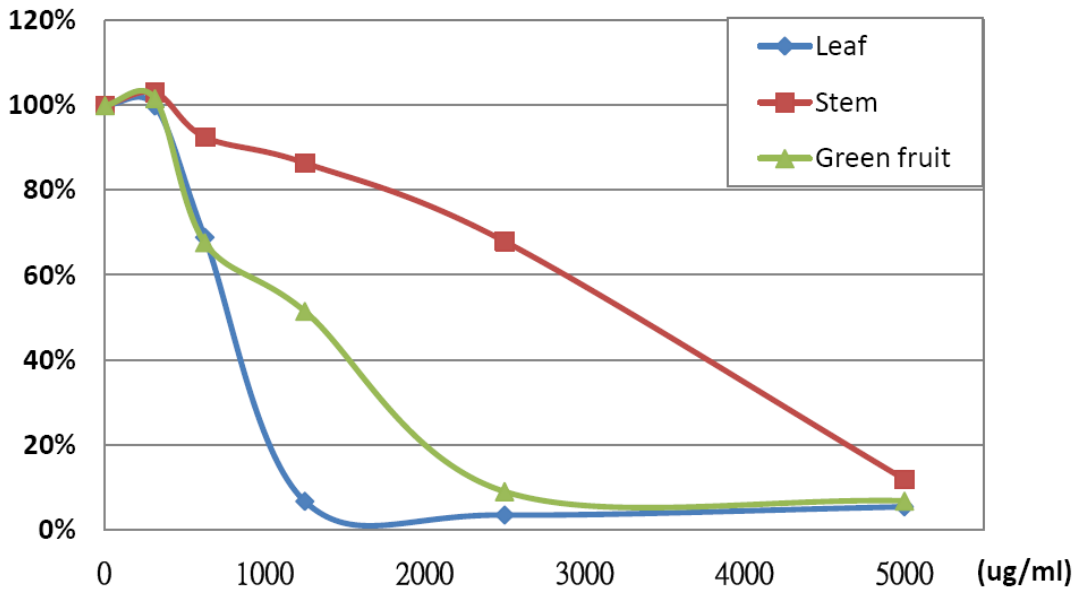
Purple fruit

Retention time (min)	8.5	15.3	16.4	28.7	32.8	34.7	
Hydrolysis (Hour)	**Del**	**Cya**	**Pet**	**Pelg**	**Peo**	**Mal**	Total
0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
0.5	144.87±25.28	N.D.	174.64±59.81	867.38±67.67	29.87±20.37	64.49±22.00	1281.23
1	202.73±28.41	N.D.	306.17±76.95	57.96±36.20	23.02±20.33	117.8±21.13	707.67
2	106.98±18.44	N.D.	188.98±58.39	N.D.	N.D.	66.95±24.75	362.91
3	49.13±16.55	N.D.	102.59±53.60	N.D.	N.D.	33.54±21.13	185.27

(ug/g)

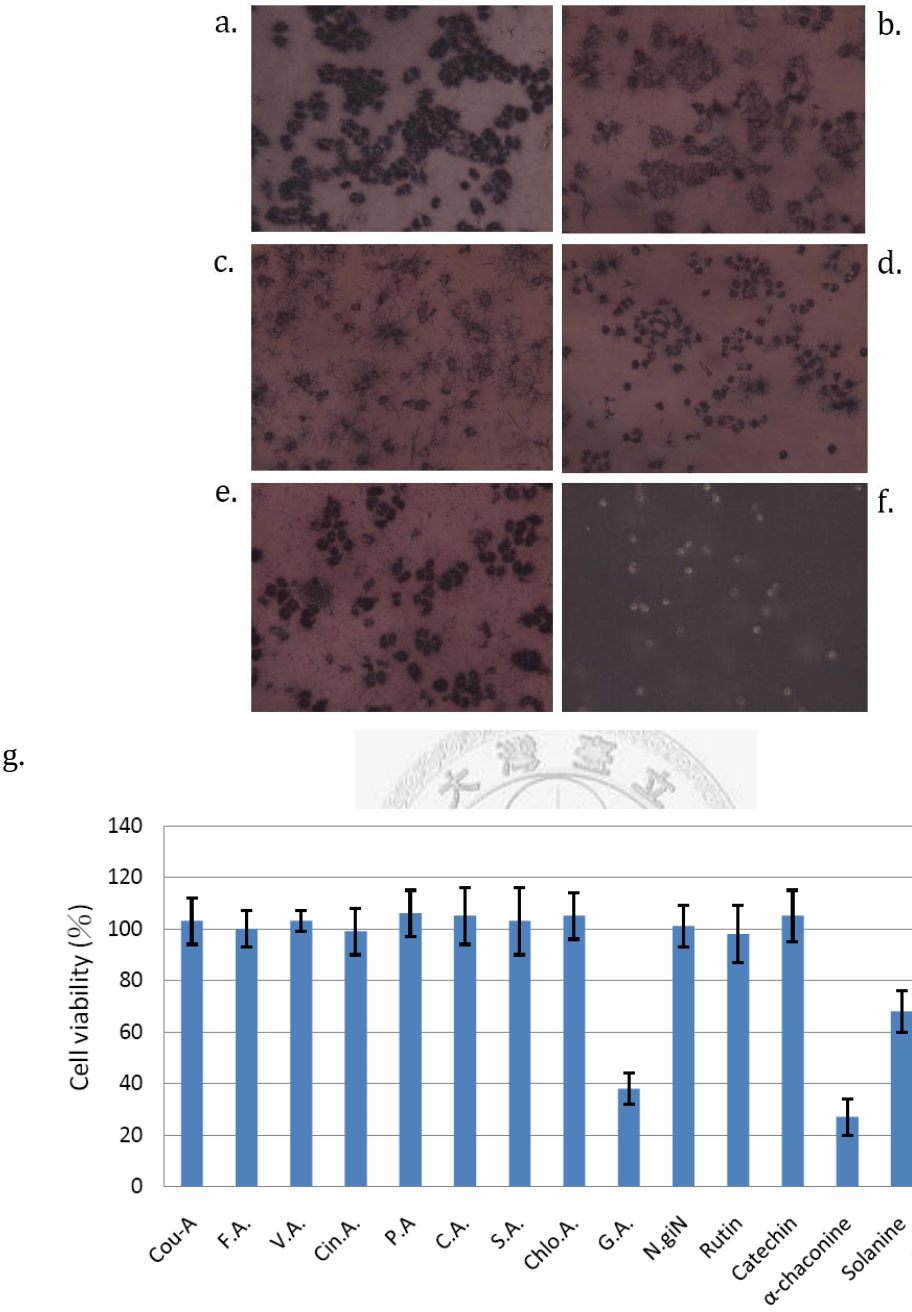
Appendix 2. Analysis of anthocyanidin content in SN extract.

Anthocyanins in SN were acid hydrolyzed to become anthocyanidin and then quantitatively analyzed by HPLC.



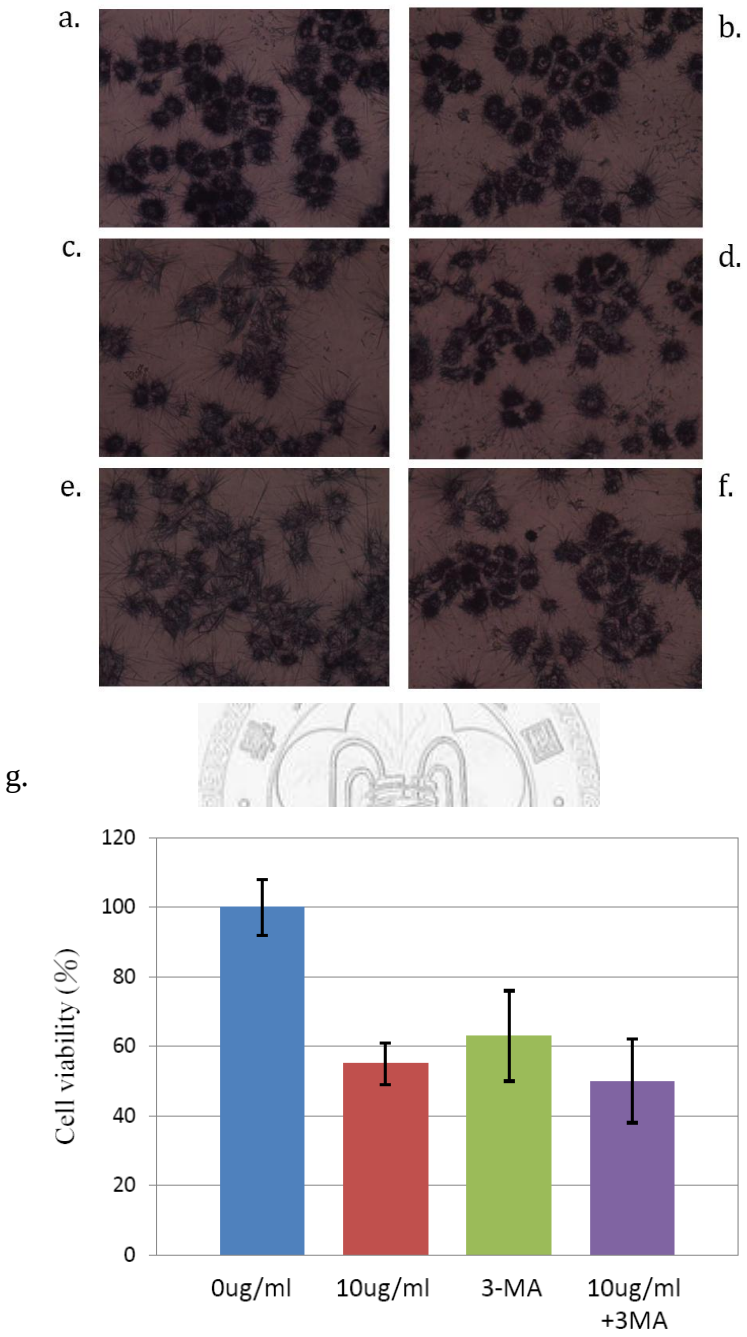
Appendix 3. SN extracts inhibited Xanthine oxidase.

SN leaf inhibit xanthine oxidase better than green fruit and stem of SN.



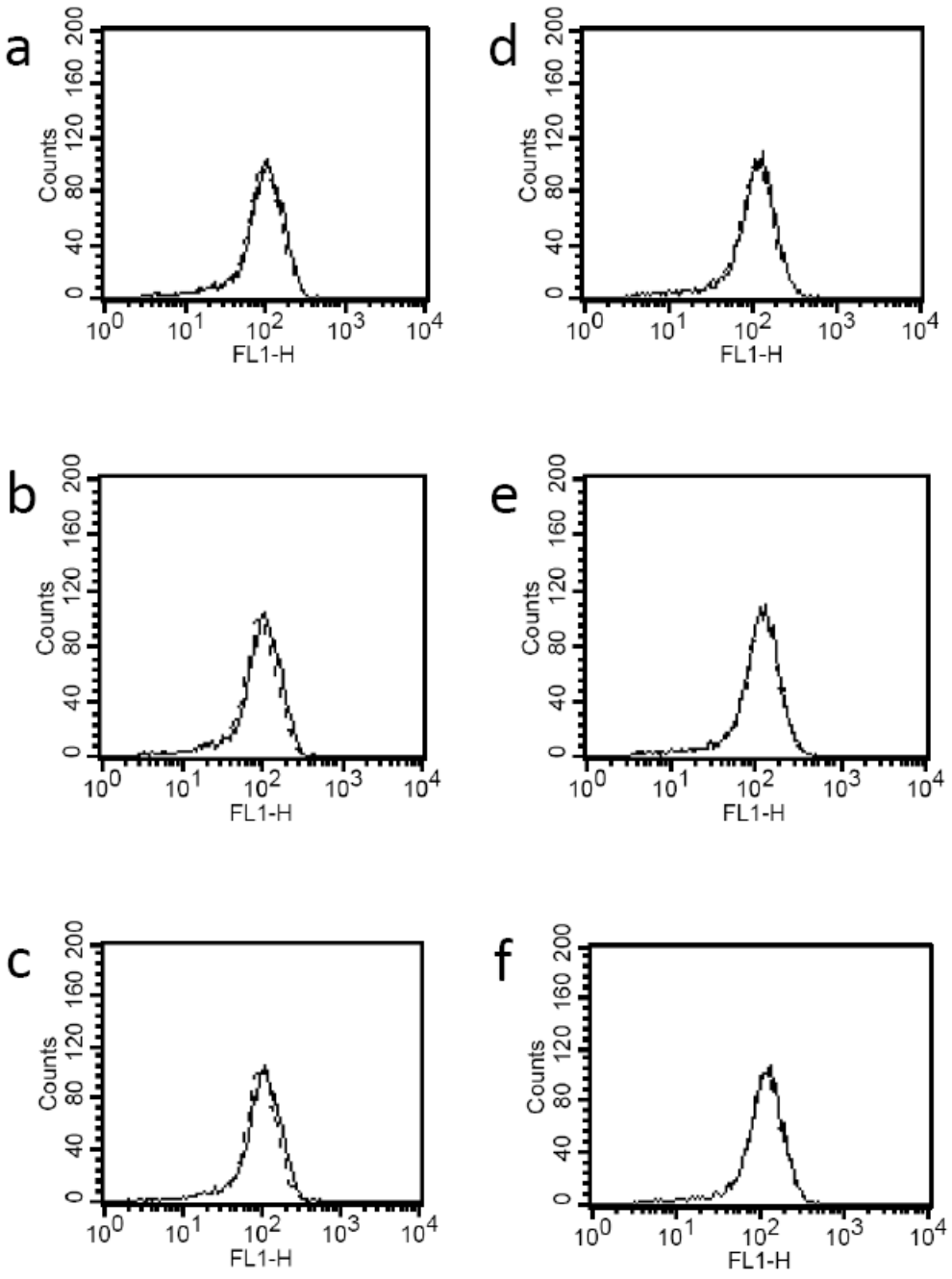
Appendix 4. AU565 were treated with 20 μg/ml of phenolic acids, polyphenols, and alkaloids.

Phenolic acids including coumaric acid (cou-A), ferulic acid (F.A.), vanillic acid (V.A.), cinnamic acid (Cin. A.), protocatechuic acid (P.A.), caffeic acid (C.A.), syringic acid (S.A.), chlorogenic acid (Chlo.A.), gallic acid (G.A.). Polyphenols including naringenin, rutin, catechin. Alkaloids including α-chaconine, solanine, diosgenin. g. showed the quantification of MTT assay. Gallic acid, α-chaconine, solanine, diosgenin were more toxic to AU565. a. to f. showed the observation of the crystal of AU565 treated by a. no treated; b. SN leaf; c. gallic acid; d. α-chaconine; e. solanine; f. d



Appendix 5. 3-MA, the autophagy inhibitor, modulated the formazan crystal reduced by SN leaf.

3-MA treated seemed block the SN leaf induced crystal decline. The crystal was observed by light-miroscopy with 400 x of original magnification. a. 0 μg/ml SN leaf as control; b. 2 mM 3-MA only; c. 10 μg/ml SN leaf; d. 10 μg/ml SN leaf +2 mM 3-MA; e. 25 μg/ml SN leaf; f. 25 μg/ml+ 2 mM 3-MA. g. is the quantification of MTT assay.



Appendix 6. SN leaf treatment did not affect the MMP and ROS in AU565 cells
a, b, c were the MMP and d, e, f were the ROS analyzed form flow cytometer. No significance was observed between control and SN leaf treated group. ---; cells were treated with SN leaf; a, d (25 μg/ml); b, e (50 μg/ml); c, f (100 μg/ml) . ─; as control, cells were not treated with SN leaf.