UNIVERSITY OF SCIENCE AND TECHNOLOGY OF HANOI

UNDERGRADUATE SCHOOL

FINAL REPORT
BACHELOR GRADUATION

By

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Project:

Evaluation of correlation between heavy metal levels – secondary metabolites contents – antioxidant activity of *Pteris vittata* using multivariate analysis

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Hanoi, August 2020

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ACKNOWLEDGEMENT

Firstly, I have taken efforts in my internship, however, it would not have been possible without the kind support and help of many individuals such as my supervisors and colleagues. Therefore, I would like to express my gratitude to all of them.

From the great support, I am highly indebted to my supervisor– Dr. Nguyen Thi Kieu Oanh for her guidance and constant supervision as well as for providing necessary information regarding this project for her support in completing the project. Moreover, PhD st. Nguyen Ngoc Lien - my lab-mate also supported me a lot in finishing the experiments and writing this thesis.

Secondly, I would like to express my gratitude towards my family, my high-school friends, and my friend in USTH - Ms. Do Doan Thu Giang for their kind co-operation and encouragement which help me in the completion of my internship for 3 months.

Finally, my thanks and appreciations also go to my colleague in developing this internship, people who have willingly helped me out with their abilities.

Thanks for all your encouragement!

Hanoi, August 14th, 2020

Pham Hai Nam

LIST OF ABBREVIATION

HM(s)	Heavy metal(s)
SM(s)	Secondary metabolite(s)
ROS	Reactive oxygen species
TPC	Total phenolic content
TFC	Total flavonoid content
DPPH	2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay
ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Assay
DMSO	Dimethyl sulfoxide
GAE	Gallic acid equivalent

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ABSTRACT

Pteris vittata has been considered to be one of hyperaccumulator plants that can grow in polluted soil. This plant adapts to metal stress by modifying their metabolism including the production of secondary metabolites that play an important role in ecological function. Additionally, this plant was used as a traditional herbal medicine with diverse therapeutic applications such as the treatment of influenza, dysentery, rheumatism, injury and scabies, abdominal pains, and diarrhea. The mechanisms of metal uptake, translocation, sequestration and tolerance in the plants have been well documented; however, the understandings of the impact of heavy metals on the production of secondary metabolites as well as their potentials in pharmacology are still limited. In this context, the objective of this study is to determine the relationship between secondary metabolite profile, total phenolic content (TPC), total flavonoid content (TFC), the heavy metal level and antioxidant properties by using DPPH and ABTS assay of *P. vittata*.

Key words: *Pteris vittata*, heavy metal level, secondary metabolites, total phenolic content, total flavonoid content, antioxidant properties, correlation network.

TÓM TẮT

Cây dương xỉ *Pteris vittata* được coi là một trong những loại cây có khả năng phát triển tốt trong vùng đất ô nhiễm kim loại nặng rất cao. Loài thực vật này thích nghi với sự ức chế kim loại bằng cách điều chỉnh quá trình trao đổi chất thông qua bộ rễ có khả năng hấp thụ kim loại của chúng bao gồm sản xuất các chất chuyển hóa thứ cấp đóng vai trò quan trọng trong các chức năng sinh thái. Loài dương xỉ này còn được dùng trong y học dân gian để trị nhiều bệnh như cúm, kiết lỵ, thấp khớp, chấn thương, ghẻ lở, đau bụng và tiêu chảy. Các cơ chế hấp thụ, chuyển vị, và chống chịu kim loại ở thực vật đã được ghi nhận đầy đủ, tuy nhiên, những hiểu biết về tác động của kim loại nặng đối với việc sản xuất các chất chuyển hóa thứ cấp cũng như tiềm năng của chúng trong y dược vẫn còn hạn chế. Trong bối cảnh đó, mục tiêu của nghiên cứu này là xác định mối quan hệ giữa chất chuyển hóa thứ cấp, tổng hàm lượng phenolic (TPC), tổng hàm lượng flavonoid (TFC), mức độ kim loại nặng và đặc tính chống oxy hóa bằng cách sử dụng thử nghiệm DPPH và ABTS của *P. vittata*.

Từ khóa: *Pteris vittata*, mức độ kim loại nặng, chất chuyển hóa thứ cấp, tổng hàm lượng phenolic, tổng hàm lượng flavonoid, đặc tính chống oxy hóa.

1. Introduction

1.1 General introduction

In Vietnam, HM pollution is becoming increasingly concern due to the massive development of industrialization and mining. Source emissions, are from industrial activities using caustic soda, chlorine, mercury waste or from the coal mines, and ore and oil, are the cause of increasing environmental pollution. Through the survey, there are many soil sources of some mining areas in Vietnam as having the heavy metal pollution. For example, in Thai Nguyen province, in 4 typical mining regions, such as Nui Hong coal mine, Trai Cau iron mine, Hich village lead - zinc mine at Tan Long commune, nui Phao tin at Ha Thuong, the content of metal, such as lead (Pb), zinc (Zn), asen (As) and cadmium (Cd), is higher many times than the permitted level. In Ha Thuong, Dai Tu, the content of asen (As) in some soil samples is higher than the permitted level about 1262 and 467 times. In Yen Lang, the content of asen (As) in soil is higher than the permitted level of Vietnam about 308 times. In Dong Hy, the content of cadmium (Cd), lead (Pb), and zinc (Zn) in the soil samples of Hich village, Tan Long is higher than other collected samples. Especially the contents of lead (Pb) in 3 samples mentioned above are 108,5; 45,1 and 51,3 ppm that are over the Vietnam regulations. The content of zine (Zn) is higher than the permitted level about 45 times. The content of asen (As) and cadmium (Cd) in 3 soil samples mentioned above is also higher than the non-polluted soil.(Chu. Thi Thu Ha, 2011)

Heavy metal pollution (HM) is known as a kind of pollution, which affects ecosystems and environmental problems. Normally, HM is toxic with plants; however, several plants could stay in the polluted areas and still grow and mature normally. They can adapt to metal stress by modifying their metabolism including the production of secondary metabolites in plant tissues. Phenolic, is known as the most important secondary metabolites, play an important and necessary role in the antioxidant in plants (Lin *et al.*, 2016). Increased phenylpropanoid metabolism and increased levels of phenolic compounds can be observed under HM stress (Sharma *et al.*, 2019). This group, especially flavonoids, can be considered as bioactive

compounds with many interesting activities such as antioxidant, antibacterial and anticancer effects (Tungmunnithum *et al.*, 2018). The question of what is the relationship between the heavy metal level, metabolites content and antioxidant activity of plants growing in these metalliferous soil thus could be interesting to answer.

Several native weeds in the above-mentioned locations were investigated previously by our research group including *P. vittata*, *P. calomelanos*, and *Eleusine indica*. In the framework of this project, we focus on the most dominant fern, *P. vittata* collected in lead and zinc mine at Hich village, Tan Long, Dong Hy, Thai Nguyen. The data of heavy metal levels in the aerial and belowground parts of 35 *P. vittata* determined by ICP-MS and metabolite profiling of these samples was done previously by our group. The main expertise of this internship is to evaluate the total phenolic and flavonoid content, antioxidant activity of samples and to use statistical analysis to reveal the relationship between the heavy metal level, the metabolites content and antioxidant properties of these plant samples by using multivariate analysis.

1.2 Literature review

Heavy metal accumulation

To classify HMs, there are 2 kinds of HMs, such as essential and non-essential. Essential HMs were explained as essential factors of living organisms, biochemical function and their maturation as well as development, such as nickel (Ni), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu). Opposing, non-essential HMs were not suitable only to stimulate the development and maturation of plants, but they are also dangerous factors to inhibit the growth of plants, namely cadmium (Cd), lead (Pb), arsenic (As), mercury (Hg), and chromium (Cr). Brief, if heavy metal concentrations overcome the threshold limit as an adverse effect on health, they will interfere with normal living functions (Martinez, 2009).

HMs disturb the redox homeostasis by arousing the free radicals and reactive oxygen species (ROS), such as singlet oxygen ($1O_2$), superoxide radicals (O_2• −), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) formations(Phaniendra, Jestadi and Periyasamy, 2015). Moreover, each type of HMs has different sites of action which interact with the plant leading to the differences between HMs of the overall visual toxic response (Nagajyoti, Lee and Sreekanth, 2010).

In HM tolerance mechanisms in plants, plants have various strategies as known as the solution to cope with the negative effects as the toxic of the metals or metalloids. To resist HM stress by "avoidance", plants can be able to restrict metal uptake or by "tolerance", plants can survive in the appearance of high internal metal concentration. Moreover, avoidance is associated with the reduction of the concentration of metal entering the cell by extracellular precipitation, biosorption to cell walls, reduced uptake, or increased efflux. On the other hand, HMs are intracellularly chelated through the synthesis of amino acids, organic acids, glutathione, or HM-binding ligands and glyoxalase systems to counter the deleterious effects caused by ROS and methylglyoxal. HM tolerance mechanisms in plants determine the coordination between the physiological and biochemical processes, including the changes in global gene expression (Watanabe and Osaki, 2002).

To apply HM hyperaccumulators for phytoremediation and phytomining, it has received considerable attention because of the possibility of exploiting for practical applications of their accumulation traits. Particularly, it is also used for improving and developing the phytoremediation technologies relating to HM contaminated soils as well as mining valuable metals from mineralized sites. It is true that the use of plants to solve the soil pollution is known as a promising factor compared to previous ways, including civil-engineering methods. Recently, there are some reviews that show the availability of the most important aspects of soil metal phytoremediation (Suman *et al.*, 2018).

Secondary metabolites and the effect of heavy metals stress on their production

All plants produce a lot of secondary metabolites as known as small biomolecules considered to be non-essential for the life of the producer organism. However, they provide many survival advantages by many ways, such as improving nutrient availability as chelating agents, protecting against environmental stressors as pigments and osmoprotectants, enhancing competitive interactions with other organisms as antibiotics, or acting as a metabolic defense mechanism as many plant flavonoid and alkaloid toxins (Breitling *et al.*, 2013).

Next, phenolic compounds are known as the most important groups of these metabolites and characterized by at least one aromatic ring (C6) combined with one or more hydroxyl groups. There are several groups of phenolic distinguished by the number of carbon atoms in conjunction with the structure of the basic phenolic skeleton, namely simple phenols, benzoic acids, phenylpropanoids and flavonoids. Phenolic is also a part of the metabolites that play an important role in plenty of functions in plants in general, moreover, most phenolics have antioxidant and antimicrobial properties. In terms of HM stresses, there are an amount of phenolic compounds as well as phenylpropanoid metabolism that could be seen. During heavy metal stress, phenolic compounds can act as metal chelators and can scavenge species of active oxygen. Moreover, enhancing phenolic link to the enhancing activity of enzymes involved in phenolic compounds metabolism was reported, suggesting that de novo synthesis of phenolic under HM stress (Tungmunnithum *et al.*, 2018). Recently, there has been a growing interest in antioxidant properties of phenolic compounds.

ROS and antioxidant activity of hyperaccumulators

Antioxidant is a factor as being responsible to the body defense system against reactive oxygen species (ROS). ROS was evaluated in damaging cell membranes and DNA, and membrane lipid peroxidation with subsequent decreases in membrane fluidity leading to oxidative damage causing cell injury, death and exacerbate the development of several age-

related chronic diseases (Goldfarb, 1993). Theoretically, heavy metals, including arsenic, lead, cadmium, and copper, present in the soil can induce the toxicity to the plants. Excess of heavy metals cause phytotoxic effects in several ways including the excessive production of reactive oxygen species (ROS). ROS was known as the factor as being highly unstable that have dual functions, such as damaging cellular components and act as an important secondary messenger for inducing plant defense system. To counteract this damage, plants are equipped with enzymatic and non-enzymatic defense mechanisms. The non-enzymatic secondary metabolites are widely produced in plants and play as antioxidants to neutralize ROS (Kasote *et al.*, 2015). Several plants are known as hyperaccumulator since they are able to grow in soils with high metals concentrations, absorbing these metals through their below ground parts, and concentrating high metals levels in their tissues. According to (Sarma, 2011), these hyperaccumulators own also a number of antioxidant defense systems to be responsible for scavenging toxic free radicals to protect themselves from oxidative stress including those caused by heavy metals(Sytar *et al.*, 2013).

2 Objectives

The objective of this internship is to unravel the relationship between secondary metabolite profile, total phenolic content (TPC), total flavonoid content (TFC), the heavy metal level and antioxidant properties of *P. vittata* collected in metalliferous soil.

To do that, research contents should be listed: (i) determination of TPC, TFC and antioxidant activities of 70 plant samples by colorimetric methods (ii) evaluate the relationship between HMs level, SMs content, and antioxidant activities of these samples by multivariate analysis.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals

To determine TPC, Gallic Acid, 6% Na_2CO_3 solution, Folin–Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). To determine TFC, 5% $NaNO_2$ solution, 10% $AlCl_3$ solution, 1M NaOH solution, ethanol 30% and eethanol were obtained from Aladdin (Shanghai, China); Rutin, Quercetin or Catechin were purchased from Vietnam National Institute of Drug Quality Control (Hanoi, Vietnam). In DPPH assay, use Ascorbic acid as standard substances and other chemicals include DMSO (Dimethyl sulfoxide) used for extracts. In ABTS assay, use Trolox as a standard substance and other chemicals include Methanol used for extracts, water and $K_2S_2O_8$ used for standard substances. These chemicals were obtained from Aladdin (Shanghai, China). Distilled water and ultrapure water were prepared using a Millipore Milli-Q purification system (Millipore GmbH, Schwalbach, Germany).

3.1.2 Instruments

96 well microtiter plate, Micropipette, Eppendorf tube and others. *Optical measuring equipment: xMark Microplate Absorbance Spectrophotometer* from BioRad (California, USA).

3.1.3 Biological materials

Plants were collected at different places in Hich village, Tan Long ward, Dong Hy district, Thai Nguyen province, Vietnam (N: 21º43’612” E: 105º51’380”) under a dry weather condition. Plant samples were identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The voucher of specimens was deposited in the Department of Life Science, University of Science and Technology of Hanoi. For sample preparation, each plant was divided into 2 parts: aerial and belowground parts. The belowground part was washed in water to remove all dust and

soil, then rinsed in distilled water. All parts were cut down into small parts, drying in the oven at 55^{o}C for 48h until the weight does not vary.

The below ground parts of plants mg of each sample) were extracted with 80% methanol (100 ml x 3 times) at room temperature in a sonic bath. The combined extracts were concentrated in vacuo to obtain crude extracts. There are 70 samples including 35 below ground parts (from S1 to S35) and 35 aerial parts (from A1 to A35).

3.2 Metabolite profiling and ionomics profiling

139 secondary metabolites detected in methanolic extract of *P. vittata* by the widely targeted metabolomics approach and their intensity of each metabolite in 70 samples were semi-quantified by using UPLC-QqQ-MS/MS (Table 2, Appendix). Table 3 in appendix shows the ICP-MS of 6 main elements of 70 samples. This data was used as input to build correlation networks between metabolites content, heavy metal levels and antioxidant properties of plant extracts.

3.3 Determination of Total Phenolic and Flavonoids content (TPC and TFC)

3.3.1 Total Phenolic content

To determine the total phenolic content by the method of using Folin-Ciocalteu reagent with gallic acid as a standard substance, the sample was weighted and dissolved in methanol. Then, take 40 µL of the diluted sample solution with 480 µL of Folin - Ciocalteu reagent, mix well for 1 minute and add 480 µL 6% Na_2CO_3 solution, mix well and incubate at 40°C for 15 minutes. The phenolic complexes were green and absorbed the wavelength λ = 765 nm. To evaluate the standard curve, mix a series of gallic acid solutions with concentrations from 0 to 1000 µg / mL following to the protocol.

3.3.2 Total Flavonoid content

To evaluate the total flavonoid content, the samples were weighted and dissolve in methanol. Then, take 240 µL of the diluted solution with 40 µL 5% $NaNO_2$ solution, mix

and incubate at 25ºC for 6 minutes, and then add 40 µL 10% $AlCl_3$ solution, mix well and incubate at 25ºC for 6 minutes and finally, add 400 µL NaOH 1M and 280 µL 30% ethanol solution, incubating for 15 minutes at 25ºC. The Flavonoids complexes were orange and absorbed the wavelength λ = 510 nm. Using the standard substances as Quercetin, mixing a series of the standard solution was with concentrations from 0 to 200 µg/mL.

3.4 Determination of antioxidant activity

3.4.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

Antioxidant activity was carried out using improved 1,1- diphenyl-2-picrylhydrazyl or DPPH assay, as described by (Kim *et al.*, 2002). In sample preparation, the samples were weighted and dissolved by DMSO at 10 mg/ml. To dilute extract for being suitable to use, the final concentrations were at 100 and 500 µg/ml, and then, mixed 10 µl sample with 190 µg DPPH solution and determined absorption band at λ = 515 nm following to the protocol. To determine the standard curve with Ascorbic acid, its solution is from 5, 10 and 15 µg Ascorbic acid per 1 ml DMSO (Galati *et al.*, 2008).

3.4.2 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Assay

Having the same purpose with DPPH assay, ABTS assay is also used for determining antioxidant assay with MeOH as a solvent. The samples were taken at 10 mg/ml and dissolved until 0.25 and 0.5 mg/ml as the final concentrations. To evaluate standard curve, its serial dilutions of compounds were from $\frac{1}{64}$, $\frac{1}{96}$, $\frac{1}{128}$, $\frac{1}{192}$, and $\frac{1}{256}$ of 50 mg Trolox per 10 ml MeOH. The absorption band is measured at 734 nm in the spectrophotometer following to the protocol.

3.5 Statistical analysis

The data of total phenolic content, total flavonoid content, and antioxidant properties using DPPH and ABTS scavenging assays were collected in one file by Microsoft Excel version 2016. All files were converted as comma-separated values (csv) files that were considered

as input data for statistical analysis. The statistical analysis was done by using RStudio version 3.5.3. (http://www.R-project.org/). Data was normalized by taking their logarithm base 10. The correlation between the heavy metal concentration, secondary metabolites content with antioxidant activities of plant were calculated by *cor* function using the default *pearson* method. Figures were built by ggplot2 R-package. (Wickham, 2016) Correlation networks were visualized though Cytoscape version 3.8.0 (Shannon *et al.*, 2003).

Correlation networks were created by making the relationship of a pair of compounds in different samples. Figure 1 was shown the example of one pair correlation as the algorithm recipes in R application of constructing linear regression of one pair samples. In both values of ABTS at 0.5 mg/ml and ABTS at 0.25 mg/ml as shown on the right, almost data of both are associated with others to create the linear regression as having R = 0.9 leading to the acceptance of this correlation. However, the linking of TPC and TFC as shown on the left is not created because the data were not arranged to construct the linear regression and R = 0.1.

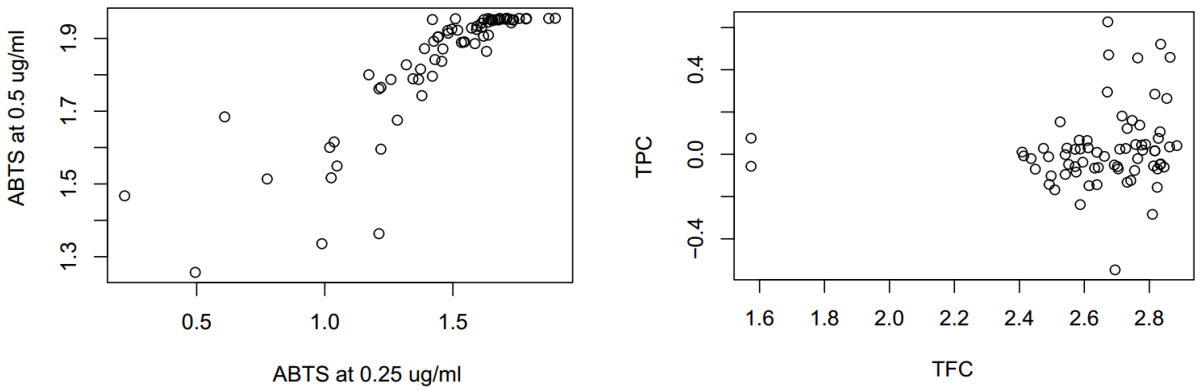

Figure 1: The example of one pair correlation

4 Results

4.1 Total phenolic content and total flavonoid content

Figure 2 shows the distribution of TFC (on the left) and TPC (on the right) data of 35 aerial parts (in orange color) and 35 belowground parts (in blue color). As can be seen from this figure, there is variation in TPC of the plant species investigated, ranging from 0.44 to 2.15 and from 0.73 to 1.92 mg GAE/g dry weight for those aerial parts and below ground parts extracts, respectively. All the samples showed remarkably low TPC (GAE < 20 mg/g dry weight). For aerial parts extracts, A11 shows the highest TPC of 2.15 mg GAE/g dry weight, while, for root extracts, S10 was the highest, with 1.92 mg GAE/g dry weight, respectively. A6 and S7 are the lowest in TPC in aerial parts and below ground parts, respectively.

The TFC of *P. vittata* were from 259.03 to 728.47 mg Quercetin/g extract in aerial parts and from 112.5 to 731.25 mg Quercetin/g extract in belowground parts. The highest content of total flavonoid was 728.47 mg Quercetin/g and 731.25 mg Quercetin/g in aerial parts / below ground parts, respectively. Using t-test, there is no significantly statistical difference between aerial parts and belowground parts in TFC (p-value > 0.05) and TPC (p-value > 0.05).

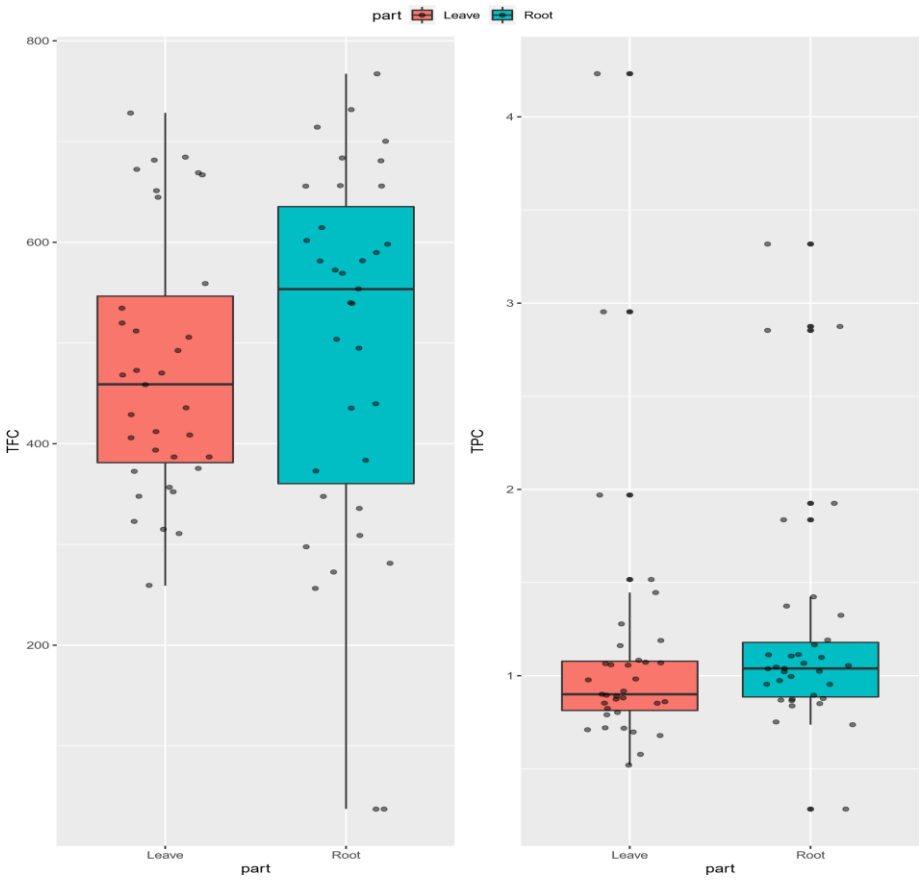

Figure 2: TFC and TPC of 70 samples: 35 aerial parts (from A1 to A35, in orange color) and 35 belowground parts (from S1 to S35, in blue color).

4.2 Antioxidant properties

4.2.1 ABTS assay

There was a large variation in the total antioxidant capacity of the aerial parts and below ground parts analyzed, as shown in Fig 3 at C_1 = 0.5 mg/ml and C_2 = 0.25 mg/ml. However, some metabolites' values of ABTS assay at C_1 = 0.5 mg/ml are almost hit the maximum (about 90% of inhibition) leading to some error numbers. Therefore, to compare the values by the exact way, it is possible to use the values of ABTS assay at C_2 =0.25 mg/ml The values ranged from 1.65 to 52.74 % and from 10.59 to 79.4 % for aerial parts and below ground parts, respectively. Highest levels of antioxidant activity of aerial parts per below ground parts extracts were obtained from A5 and S34. The lowest levels of antioxidant

activity were obtained from aerial parts /below ground parts extracts of A11 and S11. Many samples in aerial parts showed good levels of antioxidant activity when compared with below ground parts samples. Through the experiments, it was shown that the antioxidant activity of below ground parts is significantly greater than the antioxidant activity of aerial parts (p-value < 0.05) by using t-test. ABTS assay reflected the high-pigmented and hydrophilic antioxidants better than DPPH assay (Floegel *et al.*, 2011). To compare with the DPPH assay, ABTS assay confirms the antioxidant activity of DPPH assay.

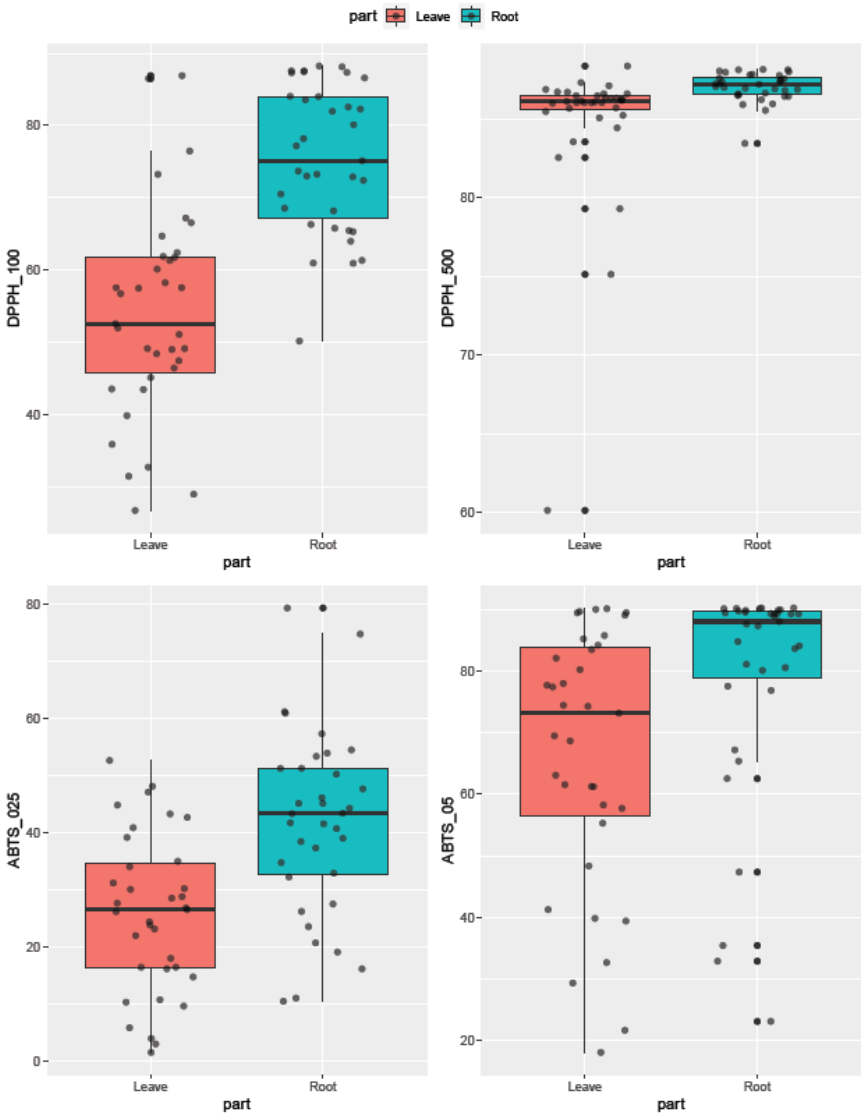

Figure 3: ABTS assay and DPPH assay of 70 samples: 35 aerial parts (from A1 to A35, in orange color) and 35 belowground parts (from S1 to S35, in blue color).

4.2.2 DPPH assay

ABTS assay was used with various advantages such as rapid, robust and accurate for systematically assessing total antioxidant capacity of extracts from plant materials on a large scale (Pisoschi *et al.*, 2016). However, combination of two antioxidant tests is recommended as each method gives different information about the antioxidant capacity of the tested extract. Therefore, DPPH assay was chosen because they are considered accurate and reliable method for assessing total antioxidant capacity (Apak *et al.*, 2007).

The data of DPPH assay were also shown at fig 3 with 2 main concentrations, such as 100 µg/ml and 500 µg/ml. At the same conclusion as ABTS assay, the data of C_1 = 100 µg/ml have more exact than the data of C_2 = 500 µg/ml due to the decreasing of errors. In the data of C_1 = 100 µg/ml, the range is from 26.76 to 86% inhibition of DPPH at aerial parts and is from 50.18 to 87.5% inhibition of DPPH. By using t-test, there is no significantly statistical difference between aerial parts and below ground parts in DPPH at 100 µg/ml. DPPH scavenging activity of all the samples of *P. vittata* was compared with standard (Ascorbic acid) by evaluating antioxidant efficiencies, known as IC50 (**table 1**). IC50 is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed. The study showed that all the samples *of P. vittata* have as compared to the standard ascorbic acid having IC50 value about 8 µg/ml the IC50 ranged from 65.39 to 186.81 µg/ml. In aerial parts, the range is from 65.39 to 186.8 µg/ml. Also, in below ground parts, the range is from 56.68 to 99.6 µg/ml. To conclude, antioxidant activity of below ground parts is greater than antioxidant activity of aerial parts.

Table 1: IC50 value of DPPH and ABTS assay of aerial parts and below ground parts

	DPPH assay (µg/ml)	ABTS assay (mg/ml)
Aerial parts	**101.328 ± 29.42**	**0.949 ± 1.397**
Below ground parts	**67.519 ± 9.951**	**0.374 ± 0.236**

4.3 Relationship between metabolite content, heavy metals level and antioxidant activities

In order to unravel whether heavy metal level has impact on the metabolism and antioxidant properties, pair to pair correlations were calculated by Pearson method. Correlation networks of the aerial parts were built by making edges between two nodes having good correlation significantly (p-value < 0.001) with the threshold of correlation coefficient 0.6 by Cytoscape 3.8.0 software. Next, correlation network of below ground parts have the correlation coefficient that is more than or equal to 0.5 and p-value is all less than 0.05 because the correlation coefficient of TPC is equal 0.5 as out of range and some metabolites in below ground parts does also not reach enough to higher value of p-value.

Both correlation networks were shown in light blue and some are special, such as metabolites link to TPC, TFC, and DPPH, that was marked by some notices, such as green is flavonoid, gray is alkaloid, brown is phenylpropanoic acid, blue is metal, red is DPPH, pink is TFC, and yellow is TPC. Some metabolites have the same values that will link together, however, there are also plenty of metabolites that do not have the similarity with others, so they just link to one or small groups of metabolites.

The simplified networks which focus mainly to the correlations containing heavy metal levels and antioxidant activities were showed in the figure 4. As being shown on above part of this figure, the DPPH activity links to TPC but does not link to TFC in 35 aerial parts. It was proven that there are lots of compounds that have a negative correlation or do not have the antioxidant activity in TFC. DPPH links to 3 different metabolites, such as metabolite **139**, metabolite **301** and metabolite **127** equivalent to 4-Coumaric acid, Isorhamnetin-3-O-rutinoside. The tolerance of the heavy metal content in plants, such as lead (Pb), zinc (Zn) and Copper (Cu), effects on the biosynthesis of secondary metabolites **213** equivalent to Saponarin. These heavy metals do not directly correlate with TPC and antioxidant activities but have indirect impact through **213**, **159**, **52**, **216** and **139** equivalents to Saponarin, Chlorogenic acid Hemihydrate, Caffeic acid, Vitexin, and 4-Coumaric acid.

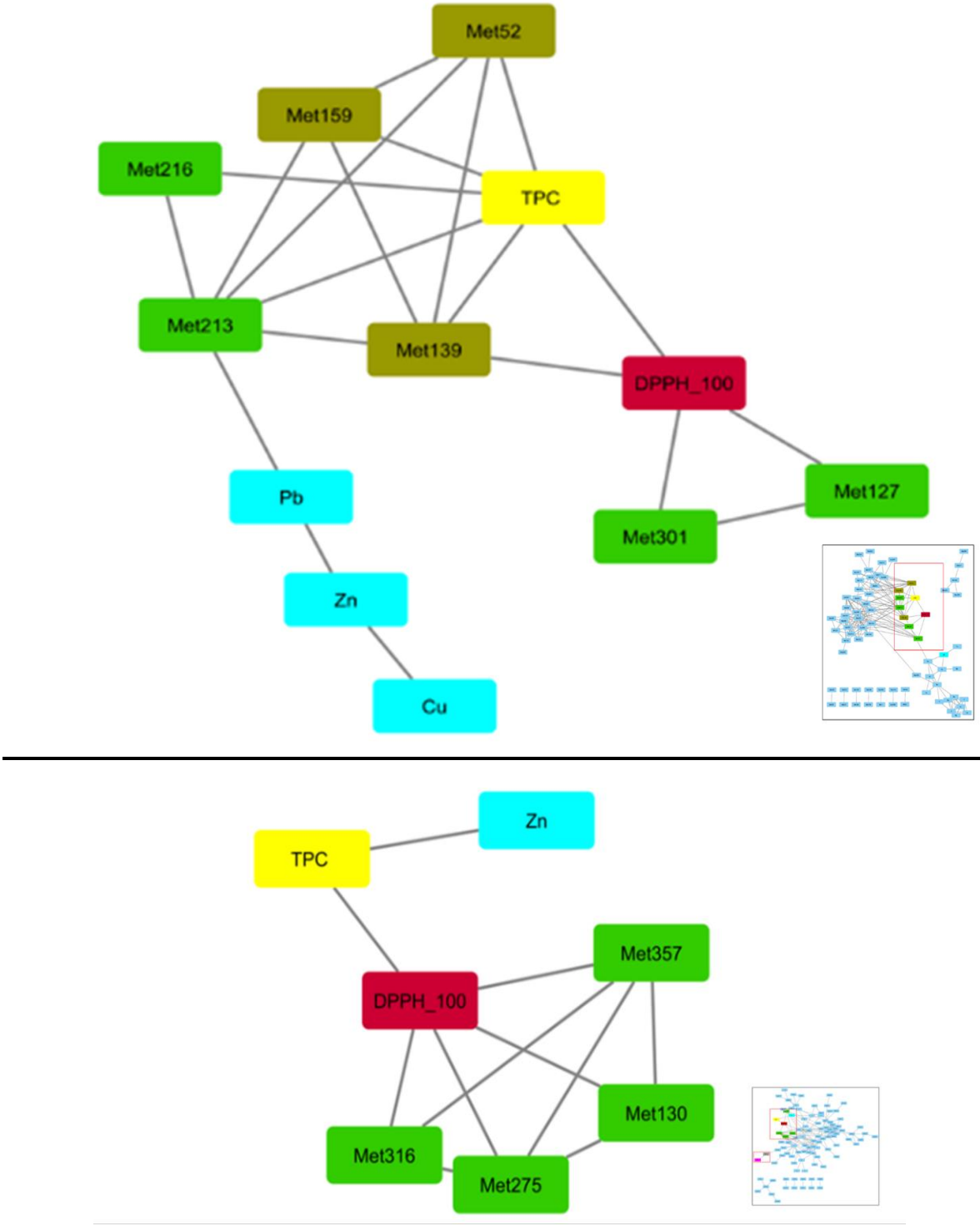

Figure 4: Correlation networks among secondary metabolites content, TPC, TFC, HM level and antioxidant activities of 35 aerial parts (above) and 35 belowground parts (below).

In belowground parts (below part of figure 4), there are 4 compounds (**357**, **130**, **275**, and **316**) that link to DPPH, such as name Quercetin-7-O-rhamnoside, Homoorietin, Catechin or Epicatechin, Quercetin-3-O-alpha-L-rhamnopyranosyl (1->2)-beta-D-glucopyranoside-7-O-alpha-L-rhamnopyranoside. Opposing with aerial parts, zinc effects directly on TPC. The different relationship between the antioxidant activity and TPC can be due to many factors. In fact, the TPC does not incorporate all the antioxidants. Also, it must be taken into account the synergism between the antioxidants in the mixture that makes the antioxidant activity not only dependent on the concentration, but also on the structure and the interaction between the antioxidants. This can explain why with similar concentrations of total phenolic, vary in their antioxidant activities. From the correlation, the bioactivity of below ground parts is greater than the bioactivity of aerial parts (shown in DPPH assay) because the below ground parts have more the metabolites that affect antioxidant activity

Moreover, ABTS assay does not link to any metabolites, so it can have some especially different mechanism with DPPH assay. Indeed, oxidative stress which can be relieved by antioxidants are caused mainly by free radicals. The antioxidants generally scavenge these radicals thus it is important to measure the free radical scavenging activity using DPPH.

5 Discussion

According to (Maneesha Singh, 2015), the total phenolic content of Pteris vittata varied from 0.21875±0.467 mg GAE/g to 1.687±1.299 and the total average was around 0.71±0.53. The flavonoid content was from 0.811±0.900 to 0.21865±0.467 mg QE/g. The IC50 of DPPH test µg/ml ranged from 260-395 µg/ml. In my experiment, TPC of aerial parts was 0.973±0.319 GAE/g and TPC of below ground parts was 1.118±0.283 GAE/g so they were greater than the total average. Next, TFC of aerial parts and below ground parts were respectively 0.476±0.129 mg QE/g and 0.507±0.156 mg QE/g compared to 6 samples of this paper, moreover, TFC data of my samples was greater than 3 per 6 samples. Therefore, the greater concentration of phenolic and flavonoid can be explained because my plant source was taken from Dong Hy, Thai Nguyen where has been known as the

heavy metal polluted areas leading to the increasing secondary metabolite generation of *P. vittata*. Also, the IC50 of aerial parts and below ground parts in my experiment were in 101.328±29.42 (µg/ml) and 67.519±9.951 (µg/ml) equivalent to DPPH test. With ABTS test, IC50 were relatively 949.046±139.788 (µg/ml) and 374.384±236.429 (µg/ml) that were out of the result range of this paper. Theoretically, DPPH assay used to evaluate the antioxidant activity of compounds that were dissolved in DMSO as a polar aprotic solvent as different as ABTS assay that used MeOH as a protic polar solvent (Ashenhurst, 2020). Each assay used to determine different metabolites (Floegel *et al.*, 2011), so that is a reason why it is essential to use more antioxidant tests to confirm and evaluate totally in antioxidant activity.

In both correlation network, there are 7 metabolites that were reported to correlate with an antioxidant activity (DPPH). In which, Quercetin-7-O-rhamnoside (Criste *et al.*, 2020) , Catechin (Bernatoniene and Kopustinskiene, 2018), Epicatechin (Pushp *et al.*, 2013) that belong to flavonoid group had been researched in previous study as having bioactivity. Moreover, 4-Coumaric acid was known as in phenylpropanoic acid group that also has antioxidant activity (Shen *et al.*, 2019).

6 Conclusion

This study aims to evaluate the correlation between heavy metal levels – secondary metabolites contents – antioxidant activity of *Pteris vittata* using multivariate analysis. The significant correlation obtained between some heavy metals, TPC, DPPH scavenging inhibition and secondary metabolites of a large number of samples and such as that phenolic content could be used to explain the mechanism of heavy metal uptake and to find out which metabolites are responsible for antioxidant activity of the studied plant. The metabolites correlate with the bioactivity to make predictions that serve the basis for performing the experiments to confirm this bioactivity of metabolites. From the metabolites as having the correlation, they were expected that they will have antioxidant activity to use for preparing medicine for human. Therefore, the results of this study may support the discovery of new medicine with antioxidant activity and further pharmacological activities from this fern and it also provides a source of useful information for the isolation of natural extracts to develop new products for natural health care.

7 References

Apak, R. *et al.* (2007) 'Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay', *Molecules*. doi: 10.3390/12071496.

Bernatoniene, J. and Kopustinskiene, D. M. (2018) 'The Role of Catechins in Cellular Responses to Oxidative Stress', *Molecules*. doi: 10.3390/molecules23040965.

Breitling, R. *et al.* (2013) 'Metabolomics for Secondary Metabolite Research', *Metabolites*. doi: 10.3390/metabo3041076.

Chu. Thi Thu Ha (2011) 'Survey on heavy metals contaminated soils in Thai Nguyen and Hung Yen provinces in Northern Vietnam', *Journal of Vietnamese Environment*. doi: 10.13141/jve.vol1.no1.pp34-39.

Criste, A. *et al.* (2020) 'Phytochemical composition and biological activity of berries and leaves from four romanian sea buckthorn (Hippophae Rhamnoides L.) varieties', *Molecules*. doi: 10.3390/molecules25051170.

Floegel, A. *et al.* (2011) 'Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods', *Journal of Food Composition and Analysis*. doi: 10.1016/j.jfca.2011.01.008.

Galati, E. M. *et al.* (2008) 'Antiinflammatory and antioxidant activity of hypericum rumeliacum Boiss. subsp. apollinis (Boiss. & Heldr.) Robson & Strid methanol extract', *Phytotherapy Research*. doi: 10.1002/ptr.2360.

Goldfarb, A. H. (1993) 'Antioxidants: Role of supplementation to prevent exercise-induced oxidative stress', *Medicine and Science in Sports and Exercise*. doi: 10.1249/00005768-199302000-00012.

Kasote, D. M. *et al.* (2015) 'Significance of antioxidant potential of plants and its relevance

to therapeutic applications', *International Journal of Biological Sciences*. doi: 10.7150/ijbs.12096.

Kim, D. O. *et al.* (2002) 'Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals', *Journal of Agricultural and Food Chemistry*. doi: 10.1021/jf020071c.

Lin, D. *et al.* (2016) 'An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes', *Molecules*. doi: 10.3390/molecules21101374.

Martinez, J. L. (2009) 'The role of natural environments in the evolution of resistance traits in pathogenic bacteria', *Proceedings of the Royal Society B: Biological Sciences*. doi: 10.1098/rspb.2009.0320.

Nagajyoti, P. C., Lee, K. D. and Sreekanth, T. V. M. (2010) 'Heavy metals, occurrence and toxicity for plants: A review', *Environmental Chemistry Letters*. doi: 10.1007/s10311-010-0297-8.

Phaniendra, A., Jestadi, D. B. and Periyasamy, L. (2015) 'Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases', *Indian Journal of Clinical Biochemistry*. doi: 10.1007/s12291-014-0446-0.

Pisoschi, A. M. *et al.* (2016) 'Antioxidant capacity determination in plants and plant-derived products: A review', *Oxidative Medicine and Cellular Longevity*. doi: 10.1155/2016/9130976.

Pushp, P. *et al.* (2013) 'Antioxidant activity and detection of (-)epicatechin in the methanolic extract of stem of Tinospora cordifolia', *Journal of Food Science and Technology*. doi: 10.1007/s13197-011-0354-8.

Sarma, H. (2011) 'Metal hyperaccumulation in plants: A review focusing on phytoremediation technology', *Journal of Environmental Science and Technology*. doi:

10.3923/jest.2011.118.138.

Shannon, P. *et al.* (2003) ‘Cytoscape: A software Environment for integrated models of biomolecular interaction networks’, *Genome Research*. doi: 10.1101/gr.1239303.

Sharma, A. *et al.* (2019) ‘Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress’, *Molecules*. doi: 10.3390/molecules24132452.

Shen, Y. *et al.* (2019) ‘Protective effects of p-coumaric acid against oxidant and hyperlipidemia-an in vitro and in vivo evaluation’, *Biomedicine and Pharmacotherapy*. doi: 10.1016/j.biopha.2018.12.074.

Suman, J. *et al.* (2018) ‘Phytoextraction of heavy metals: A promising tool for clean-up of polluted environment?’, *Frontiers in Plant Science*. doi: 10.3389/fpls.2018.01476.

Sytar, O. *et al.* (2013) ‘Heavy metal-induced oxidative damage, defense reactions, and detoxification mechanisms in plants’, *Acta Physiologiae Plantarum*. doi: 10.1007/s11738-012-1169-6.

Tungmunnithum, D. *et al.* (2018) ‘Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview’, *Medicines*. doi: 10.3390/medicines5030093.

Watanabe, T. and Osaki, M. (2002) ‘Mechanisms of adaptation to high aluminum condition in native plant species growing in acid soils: A review’, *Communications in Soil Science and Plant Analysis*. doi: 10.1081/CSS-120003885.

Wickham, H. (2016) *ggplot2 Elegant Graphics for Data Analysis (Use R!)*, *Springer*. doi: 10.1007/978-0-387-98141-3.

Ashenhurst, J. (2020). *https://www.masterorganicchemistry.com/*. From master organic chemistry: "https://www.masterorganicchemistry.com/2012/04/27/polar-protic-polaraprotic-nonpolar-all-about-solvents/"

Maneesha Singh, K. M. (2015). Phytochemical Evaluation and Antioxidant Activity of Different Samples of Pteris Vittata in Doon Valley, Uttarakhand Region. *INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE*.

8 Appendices

Standard curve of TPC, TFC, antioxidant using DPPH and ABTS assay

Phenolic are one major class of secondary metabolites that have been widely studied due to their well-known antioxidants. Total phenolic content was estimated by the Folin-Ciocalteu colorimetric method, using gallic acid as standard phenolic compound. A linear calibration curve of gallic acid, in range 0 to 200 µg/mL with R^2 value of 0.9952 with the equation y = 0.002x + 0.1054, was constructed (Fig.1). The TPC was expressed as gallic acid equivalents (GAE) in milligram per gram dry material.

Flavonoid are well known antioxidant constituents of plants and possess a broad spectrum of chemical and biological activity, including radical scavenging properties. The TFC of *P. vittata* were quantified. The regression equation of the standard curve of quercetin was y = 0.0005x - 0.0045 with R^2 = 0.9903. The results showed that the linear relationship was good in the detection range.

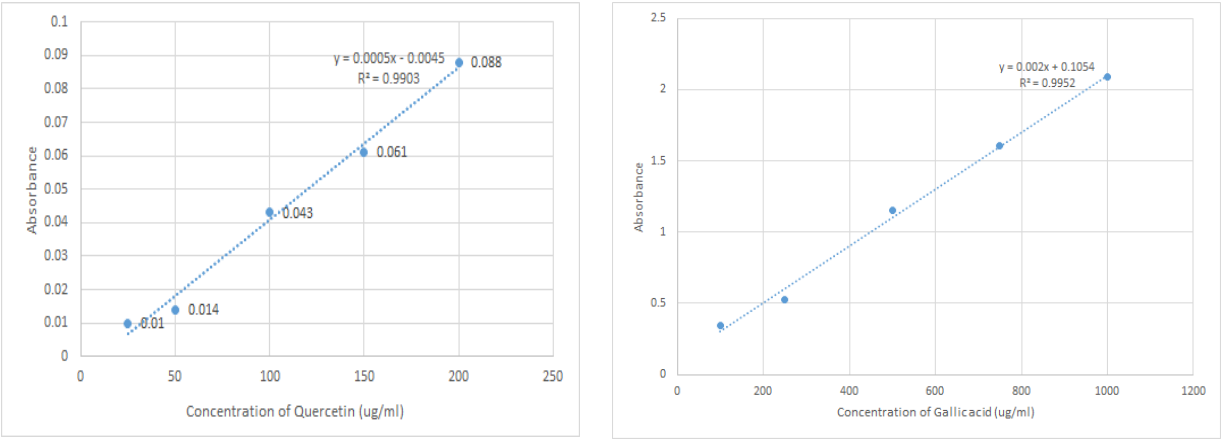

Figure 5: Calibration curve for determination of TFC (on the left) and TPC (on the right). Correlation coefficient R^2 = 0.9903 and 0.9952 shows the linearity of data.

The improved ABTS method was used to determine the antioxidant capacity for the plant species examined in this work. The concentration response curves for ABTS, as a function of separately prepared stock solutions of 5 mg/ml Trolox standards $(\frac{1}{64}, \frac{1}{96}, \frac{1}{128}, \frac{1}{192}, \frac{1}{256}, \frac{1}{384}, \frac{1}{512}, \frac{1}{768}, \frac{1}{1024})$ are shown in Fig.3 in term of Trolox equivalent to antioxidant capacity (TEAC, µmol trolox equivalents per gram dry weight of plant).

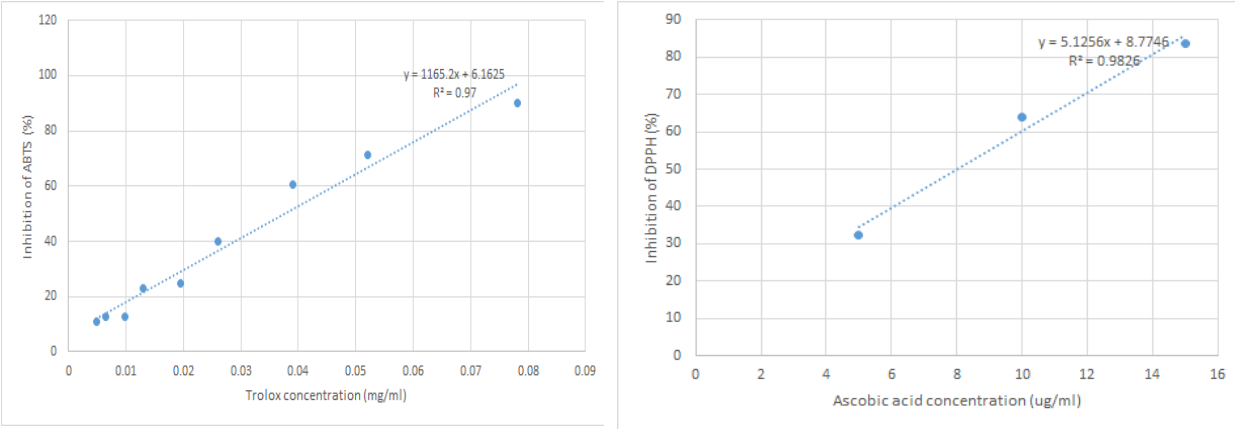

Figure 6: Calibration curve for the determination of ABTS and DPPH scavenging inhibition. Correlation coefficients $R^2 = 0.97$ with ABTS assay and $R^2 = 0.9826$ with DPPH assay. Show the linearity of data.

Table 2: 139 secondary metabolites detected in methanol extract of *P. vittata*

No.	Metabolite ID	Name	Group
1	104	Spermine	Alkaloids and derivatives
2	173	Scoulerin	Alkaloids and derivatives
3	33	Harmaline hydrochloride dihydrate	Alkaloids and derivatives
4	37	1-Methylhistamine dihydrochloride	Alkaloids and derivatives
5	255	1,3-Dimethylurate	Alkaloids and derivatives
6	256	3-Methylxanthine	Alkaloids and derivatives
7	7	Serotonin hydrochloride	Alkaloids and derivatives
8	24	3-(2-Aminoethyl)indole Hydrochloride	Alkaloids and derivatives
9	71	3-Indoleacetic acid	Alkaloids and derivatives
10	228	3-Indoxylsulfate potassium salt	Alkaloids and derivatives
11	269	Indole-3-carboxylic acid	Alkaloids and derivatives
12	299	Melatonin	Alkaloids and derivatives
13	344	2i_Indole-3-carboxyaldehyde_Indole-3-aldehyde	Alkaloids and derivatives
14	48	Amantadine hydrochloride	Alkaloids and derivatives
15	150	N-Acetyl putrescine hydrochloride	Alkaloids and derivatives

16	215	Solasodine	Alkaloids and derivatives
17	13	Caffeine,Anhydrous	Alkaloids and derivatives
18	324	2i_Theophylline,anhydrous_1,7-Dimethylxanthine	Alkaloids and derivatives
19	253	Trigonelline hydrochloride	Alkaloids and derivatives
20	274	Betaine	Alkaloids and derivatives
21	12	Anthranilic acid	Benzoic acids and derivatives
22	30	4-Aminobenzoic acid	Benzoic acids and derivatives
23	99	3,4-Dihydroxybenzoic acid	Benzoic acids and derivatives
24	153	3-Hydroxyanthranilic acid	Benzoic acids and derivatives
25	178	Vanillin	Benzoic acids and derivatives
26	183	Syringaldehyde	Benzoic acids and derivatives
27	229	2,5-Dihydroxy benzoic acid	Benzoic acids and derivatives
28	230	Hippuric Acid	Benzoic acids and derivatives
29	231	Salicylic Acid	Benzoic acids and derivatives
30	258	4-Hydroxy-3-methoxybenzoic acid	Benzoic acids and derivatives
31	260	5-Methoxysalicylic acid	Benzoic acids and derivatives
32	294	o-Anisic Acid	Benzoic acids and derivatives
33	347	DL-Mandelic acid	Benzoic acids and derivatives
34	352	4-Hydroxybenzoate Na	Benzoic acids and derivatives

35	147	4-Methylumbelliferyl acetate	Coumarins and derivatives
36	296	Daphnetin	Coumarins and derivatives
37	297	6,7-Dihydroxycoumarin	Coumarins and derivatives
38	298	Esculin sesquihydrate	Coumarins and derivatives
39	323	4-Methylumbelliferone	Coumarins and derivatives
40	123	Pelargonin chloride	Flavonoids
41	126	Keracyanin Chloride	Flavonoids
42	271	Cyanidin-3-O-rhamnoside chloride	Flavonoids
43	281	Cyanidin-3-O-alpha-arabinopyranoside	Flavonoids
44	282	Peonidin-3-O-alpha-arabinopyranoside	Flavonoids
45	283	Petunidin-3-O-beta-glucopyranoside	Flavonoids
46	285	Cyanidin-3-O-(2"-O-beta-xylopyranosyl-beta-glucopyranoside)	Flavonoids
47	286	Peonidin-3,5-O-di-beta-glucopyranoside	Flavonoids
48	287	Delphinidin-3-O-(2"-O-beta-xylopyranosyl-beta-glucopyranoside)	Flavonoids

49	288	Cyanidin-3-O-(2"-O-beta-xylopyranosyl-beta-glucopyranoside)-5-O-beta-glucopyranoside	Flavonoids
50	289	Cyanidin-3-O-[6"-O-(E-p-coum)-2"-O-(beta-xylopyranosyl)-beta-glucopyranoside]-5-O-beta-glucopyranoside	Flavonoids
51	290	Petunidin-3-O-(6"-O-(4"'-O-E-coum)-alpha-rhamnopyranosyl-beta-glucopyranosyl)-5-O-beta-glucopyranoside	Flavonoids
52	304	Cyanidin-3,5-di-O-glucoside	Flavonoids
53	307	Ideain	Flavonoids
54	312	Malvidin hex	Flavonoids
55	314	Peonidin hex	Flavonoids
56	308	Maritimein	Flavonoids
57	203	2',6'-Dihydroxy-4-Methoxychalcone-4'-O-Neohesperidoside	Flavonoids
58	309	Eriodictyol-7-O-glucoside	Flavonoids
59	202	4-Deoxyphloridzin	Flavonoids
60	210	Neohesperidin dihydrochalcone	Flavonoids

61	211	Phloridzin	Flavonoids
62	279	Procyanidin C1	Flavonoids
63	316	(+)-Catechin or (+)-Epicatechin	Flavonoids
64	120	Eriodictyol	Flavonoids
65	122	Naringenin	Flavonoids
66	131	Naringenin-7-O-glucoside	Flavonoids
67	191	Hesperetin	Flavonoids
68	204	Flavanomarein	Flavonoids
69	205	Hesperidin	Flavonoids
70	208	Neoeriocitrin	Flavonoids
71	209	Neohesperidin	Flavonoids
72	212	Poncirin	Flavonoids
73	175	(+-)-Taxifolin	Flavonoids
74	124	Apigenin-7-O-glucoside	Flavonoids
75	125	Rhoifolin	Flavonoids
76	130	Homoorietin	Flavonoids
77	206	Luteolin-3',7-di-O-glucoside	Flavonoids
78	207	Luteolin-4'-O-glucoside	Flavonoids
79	213	Saponarin	Flavonoids

80	216	Vitexin	Flavonoids
81	303	2i_Fortunellin_Linarin	Flavonoids
82	127	Isorhamnetin-3-O-glucoside	Flavonoids
83	128	Robinin	Flavonoids
84	129	Kaempferol-3-O-glucoside	Flavonoids
85	133	Peltatoside	Flavonoids
86	134	Hyperoside	Flavonoids
87	135	Quercetin-3-O-glucose-6''-acetate	Flavonoids
88	141	Kaempferol-7-O-alpha-L-rhamnoside	Flavonoids
89	220	Kaempferol-3-Rhamnoside-4''-Rhamnoside,-7-Rhamnoside	Flavonoids
90	221	Kaempferol-3-O-alpha-L-arabinoside	Flavonoids
91	222	Kaempferol-3-Glucuronide	Flavonoids
92	223	Myricetin-3-Galactoside	Flavonoids
93	224	Myricetin-3-Xyloside	Flavonoids
94	225	Quercetin-3-Glucuronide	Flavonoids
95	275	Quercetin-3-O-alpha-L-rhamnopyranosyl(1->2)-beta-D-	Flavonoids

		glucopyranoside-7-O-alpha-L-rhamnopyranoside	
96	284	Quercetin-3,4'-O-di-beta-glucopyranoside	Flavonoids
97	300	Kaempferol-dihex	Flavonoids
98	301	Isorhamnetin-dihex	Flavonoids
99	302	Kaempferol-3-O-beta-D-glucoside-7-O-alpha-L-rhamnoside	Flavonoids
100	305	Kaempferol dihex	Flavonoids
101	306	Kaempferol hex	Flavonoids
102	310	Quercetin hex	Flavonoids
103	311	Quercetin hex	Flavonoids
104	313	Syringetin hex	Flavonoids
105	357	Quercetin-7-O-rhamnoside	Flavonoids
106	358	Rutin	Flavonoids
107	121	Genistein	Isoflavonoids
108	132	Puerarin	Isoflavonoids
109	214	Sissotrin	Isoflavonoids
110	251	Acetaminophen	Phenols

111	4	(R)-(-)-Phenylephrine hydrochloride	Phenols
112	257	3-Hydroxymandelic acid	Phenols
113	119	Catechol	Phenols
114	136	Capsaicin	Phenols
115	137	Dihydrocapsaicin	Phenols
116	138	Nordihydrocapsaicin	Phenols
117	239	Homovanillic acid	Phenols
118	98	4-Nitrophenol	Phenols
119	190	4-Methoxycinnamic acid	Phenylpropanoic acids and derivatives
120	322	2 or 3-Methoxycinnamic acid	Phenylpropanoic acids and derivatives
121	186	3,4-Dimethoxycinnamic acid	Phenylpropanoic acids and derivatives
122	187	3,5-Dimethoxycinnamic acid (predominantly trans)	Phenylpropanoic acids and derivatives
123	52	Caffeic acid	Phenylpropanoic acids and derivatives
124	95	2-Hydroxycinnamic acid, predominantly trans	Phenylpropanoic acids and derivatives

125	139	4-Coumaric acid	Phenylpropanoic acids and derivatives
126	140	Rosmarinic acid	Phenylpropanoic acids and derivatives
127	159	Chlorogenic acid Hemihydrate	Phenylpropanoic acids and derivatives
128	188	m-Hydroxycinnamic acid	Phenylpropanoic acids and derivatives
129	194	trans-3,5-Dimethoxy-4-hydroxycinnamaldehyde	Phenylpropanoic acids and derivatives
130	276	1-O-b-D-glucopyranosyl sinapate	Phenylpropanoic acids and derivatives
131	327	Ferulic acid	Phenylpropanoic acids and derivatives
132	236	DL-p-Hydroxyphenyllactic acid	Phenylpropanoic acids and derivatives
133	238	DL-3-Phenyllactic acid	Phenylpropanoic acids and derivatives
134	1	4-Hydroxy-3-methoxycinnamaldehyde	Phenylpropanoic acids and derivatives
135	291	E-3,4,5'-Trihydroxy-3'-glucopyranosylstilbene	Stilbenoids

136	292	E-4,5'-Dihydroxy-3-methoxy-3'-glucopyranosylstilbene	Stilbenoids
137	64	Gibberellin A4	Terpenoids
138	162	Gibberelin A3	Terpenoids
139	155	Abscisic acid	Terpenoids

Table 3: ICP-MS of 6 elements of 70 samples

Code	Cu	Pb	Cd	Zn	Hg	As
A1	59.628	953.172	5.9	4144.053	26.462	5626.387
S1	139.507	23809.031	88.366	31818.661	6.437	3218.153
A2	48.652	470.166	1.733	990.439	30.277	5588.422
S2	67.515	6971.838	4.981	11767.716	13.589	278.662
A3	64.879	823.889	2.843	883.155	41.72	9554.867
S3	44.174	3236.553	1.345	2493.67	24.078	1185.614
A4	50.808	181.17	1.035	328.86	13.589	6718.871
S4	58.908	1573.063	2.019	1063.27	9.536	7504.516
A5	45.404	265.107	1.206	905.463	23.84	2375.399
S5	36.835	2905.403	1.683	3779.495	13.35	160.506
A6	58.859	458.107	1.279	641.041	30.038	8546.527
S6	44.067	2206.013	1.078	1241.671	12.635	2055.749
A7	44.936	531.053	1.441	801.673	30.515	5520.219
S7	50.635	3737.098	2.261	3922.925	17.403	535.051
A8	44.062	308.153	1.358	653.137	25.986	5360.742
S8	48.506	4950.558	2.695	6521.427	14.066	1384.838
A9	49.414	499.618	1.307	797.016	25.27	5326.458

S9	69.908	4647.223	3.127	3563.462	43.628	899.729
A10	68.079	406.167	2.474	568.054	23.363	7169.356
S10	71.265	5546.246	5.033	3809.366	9.059	798.165
A11	38.562	203.645	0.792	610.731	21.933	3146.918
S11	42.218	2464.754	2.447	4963.409	11.443	926.53
A12	62.32	539.419	1.562	1122.457	34.807	6665.076
S12	59.772	6434.883	2.034	6508.456	15.496	365.756
A13	67.767	507.105	4.102	1640.306	25.509	3105.085
S13	124.445	6066.249	10.7	6931.423	46.012	1334.09
A14	85.014	2624.272	8.409	3798.529	19.31	12480.673
S14	38.245	3954.005	4.476	3427.072	14.066	1286.437
A15	33.087	174.654	0.71	354.205	4.299	2330.971
S15	105.86	2195.635	2.455	1673.529	8.431	6027.458
A16	65.149	265.753	1.228	742.75	46.25	7859.407
S16	73.197	3484.498	2.771	2826.405	16.45	4859.375
A17	67.442	257.309	2.344	2627.892	22.648	8134.04
S17	77.941	2935.486	9.621	5981.385	24.078	6095.088
A18	39.829	216.814	1.355	721.73	28.846	2012.552
S18	57.708	1570.261	5.274	3713.286	40.29	2944.491

A19	3.78	31.621	0.668	62.754	26.968	264.814
S19	2.709	186.657	0.882	159.553	27.359	72.579
A20	30.028	105.515	0.747	307.32	15.019	3574.732
S20	64.956	1547	2.488	1295.668	9.774	6756.504
A21	56.611	237.415	1.424	1082.864	15.973	5437.397
S21	64.142	1826.001	1.382	2979.513	33.376	762.052
A22	247.124	469.877	3.516	919.951	416.545	118.881
S22	56.264	2026.864	1.519	2707.254	15.973	589.545
A23	73.771	164.524	0.954	1137.745	17.165	3666.407
S23	63.503	1146.783	2.46	6657.643	2.146	3006.806
A24	61.498	288.888	1.115	989.357	11.92	7089.703
S24	99.093	2644.327	2.658	3849.948	6.198	1140.495
A25	70.134	128.446	1.163	1207.995	46.25	4398.998
S25	83.853	1832.326	2.348	2632.764	22.41	3756.591
A26	5.928	35.956	0.821	132.22	25.293	264.949
S26	2.464	140.351	0.847	200.619	25.349	110.973
A27	61.562	125.428	0.914	626.26	10.49	4025.72
S27	50.964	1759.327	3.771	9376.636	2.622	1544.709
A28	5.52	23.334	0.751	72.71	27.806	223.924

S28	4.711	381.77	0.995	205.92	26.466	99.047
A29	39.862	139.327	1.288	359.248	11.92	3172.069
S29	51.099	2437.114	4.444	2190.265	2.384	1759.472
A30	61.063	284.845	1.754	784.934	13.112	6723.276
S30	59.65	2172.127	3.194	969.934	3.814	3544.937
A31	42.191	157.657	0.992	374.82	7.39	4929.299
S31	80.878	2108.186	5.535	8090.215	10.251	3281.414
A32	77.32	266.335	1.757	960.783	11.682	5863.626
S32	196.086	2286.237	3.251	5097.226	20.979	893.685
A33	61.355	207.442	1.373	661.09	12.158	5790.848
S33	70.09	1913.058	1.674	3969.868	3.814	686.861
A34	77.36	298.421	2.211	1277.341	33.376	2022.184
S34	57.97	1572.804	3.477	3289.293	26.224	857.829
A35	70.288	355.181	2.368	854.731	16.211	7363.655
S35	77.138	2570.672	3.4	2913.832	13.827	6374.016

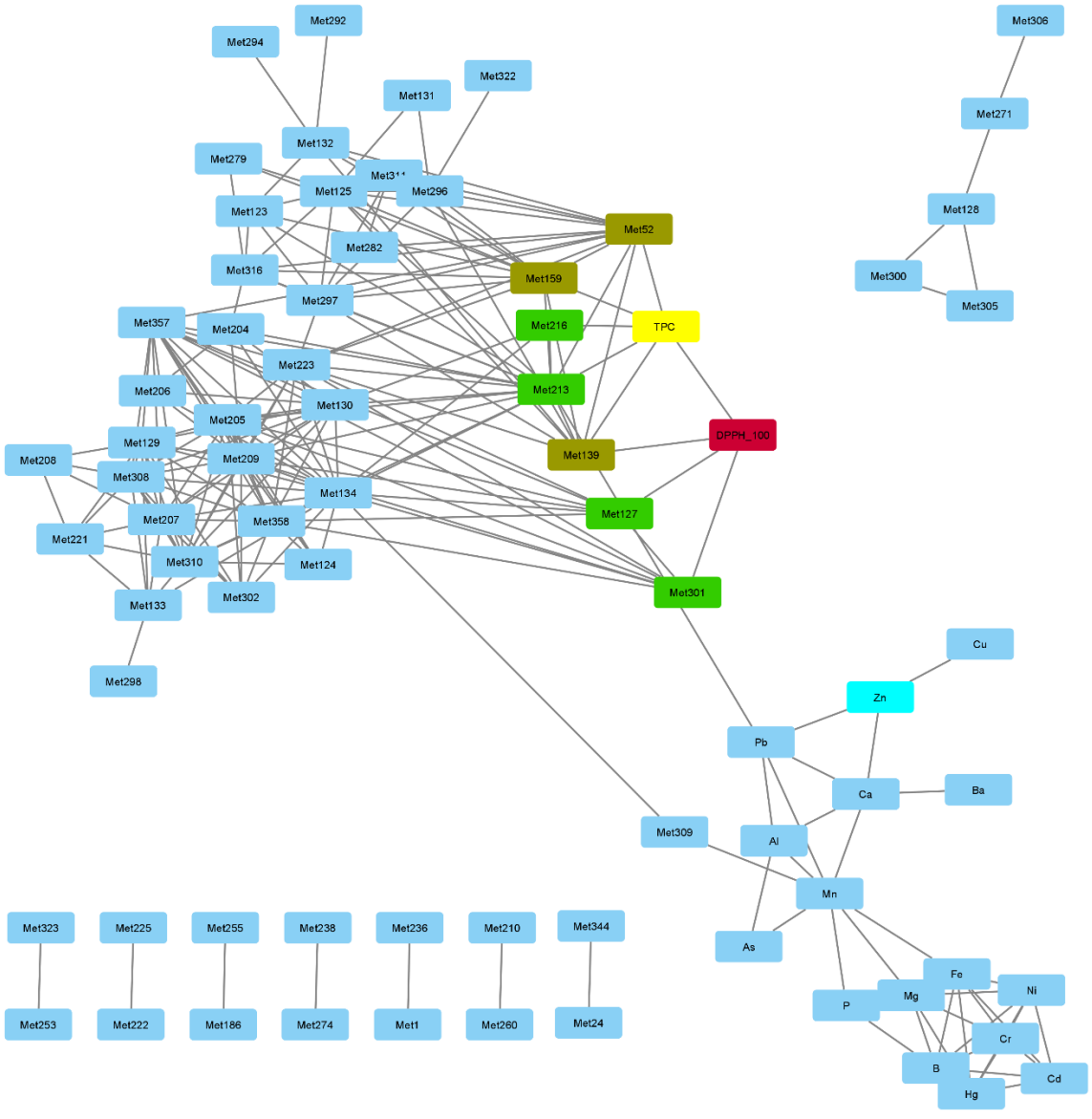

Figure 8: Correlation between TPC, TFC, DPPH, ABTS and metabolites of the aerial parts samples

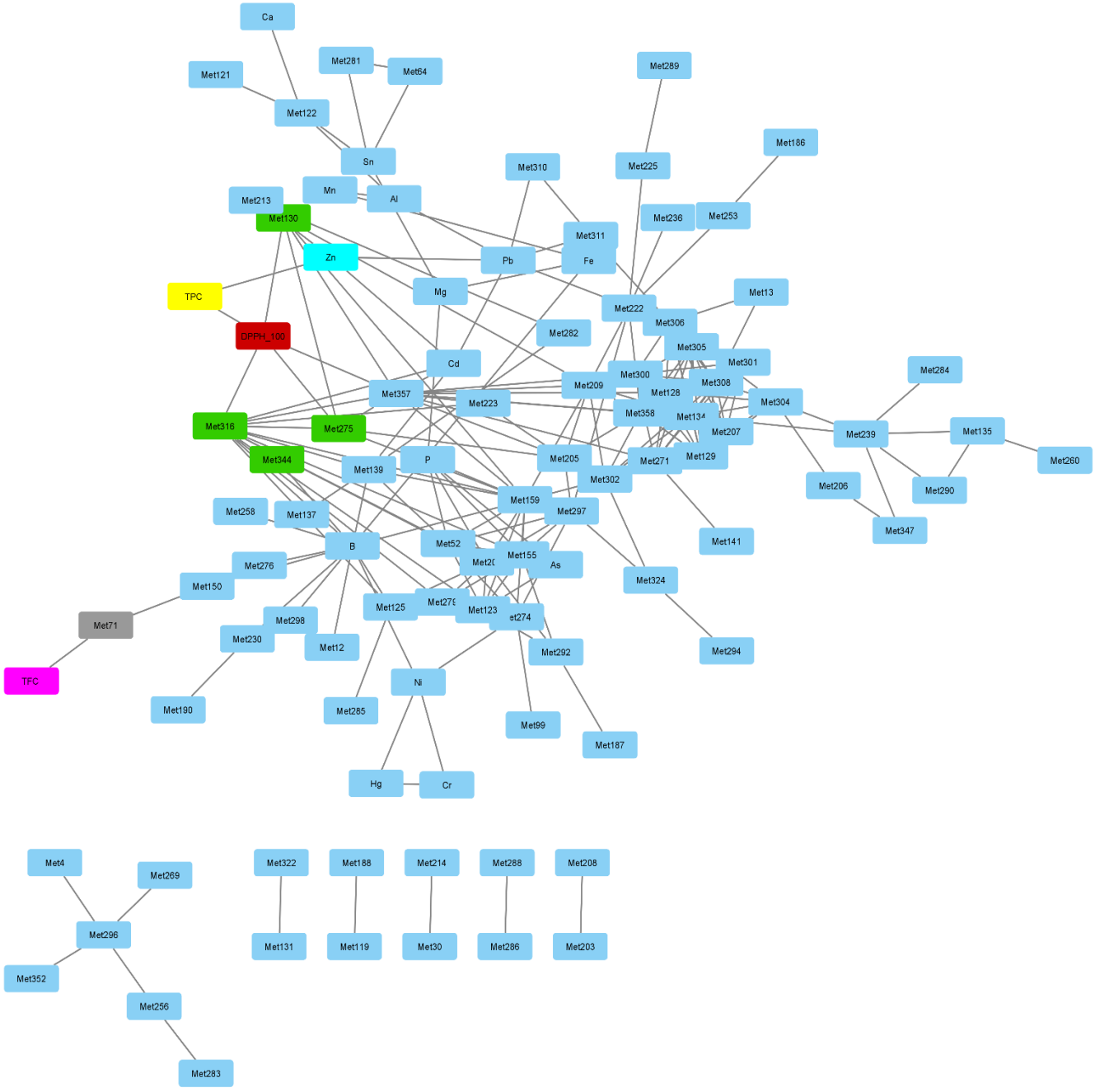

Figure 8: Correlation between TPC, TFC, DPPH, ABTS and metabolites of the below ground parts samples