# FOLIAR N AND POLYPHENOL CONTENT IN SELECTED TREE SPECIES IN BUKIT TUPAI MOUNT KINABALU

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# THIS DISSERTATION IS SUBMITTED AS A PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

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1. SUPERVISOR (MADAM LUIZA MAJUAKIM)



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## ABSTRACT

This study was conducted to determine the interaction of phenolic and nitrogen in selected tree species in nutrient deficient forest of Mount Kinabalu. Leaves of nine species were collected in three replicates. The total phenolic content of leaves sample were determined by using Folin-Ciocalteu colorimetric method with slight modifications and using tannic acid as standard. Total nitrogen and carbon content in leaves were determined using Elementar vario MAX CN analyzer. Total phenolic content was significantly different (p < 0.001) between species. Total phenolic content in leaves ranged between 1.46 to 4.84 % d.w. TAE. Ascarina philippinensis had the lowest phenolic content with 1.46 ± 0.29 % d.w. TAE, while, Myrica javanica had the highest phenolic content with 4.84 ± 0.32 % d.w. TAE. Podocarp species showed an increase in total phenolic content except Dacrycarpus imbricatus. A significant negative correlation between total phenolic and total nitrogen content was obtained  $(r = -0.8030 \text{ and } R^2 = 0.6449)(p<0.01)$ . C:N ratio in leaves of different tree species was significantly different (p<0.05). A significant negative correlation was obtained between total C:N ratio to total nitrogen content (r = - 0.9600;  $R^2 = 0.9224$ ) (p<0.01). C:N ratio indicates leaf quality and degradability. High C:N ratio suggests low quality litter that reduces degradability. Litter quality determines rate of decomposition because quality of litter changes as a result of carbon and nitrogen utilization by decomposer. Low quality litter may in turn cause nitrogen deficit, thereby increases the production of phenolic in plants. Nutrient limited plants suffer from low growth rate and have higher concentration of carbon-based secondary metabolites. The negative correlation in total phenolic with nitrogen in leaves agreed with the carbon-nutrient balance hypothesis. The hypothesis suggests that resource availability affects production of secondary compounds. Plant species producing high phenolic content may possess different strategies to adapt in resource poor environment to overcome resource limitation.



## ABSTRAK

# Kajian Kandungan Nitrogen and polifenol dalam spesies pokok terpilih di Bukit Tupai, Gunung Kinabalu.

Kajian ini bertujuan untuk menentukan interaksi antara fenolik dan nitrogen dalam beberapa spesies pokok di hutan yang kurang nutrien di Gunung Kinabalu. Daun daripada sembilan spesies pokok (tiga individu setiap spesies) telah dikutip. Kandungan fenolik dalam sampel daun telah ditentukan dengan menggunakan kaedah Folin-Ciocalteu yang diubahsuai dan menggunakan asid tannik sebagai piawai. Kandungan karbon dan nitrogen telah diuji dengan peralatan Elementar vario MAX CN. Kandungan fenolik dalam sampel daun adalah signifikan di antara spesies (p<0.001). Kandungan fenolik berada dalam julat 1.46 ke 4.84 % d.w. TAE. Ascarina philippinensis mengandungi jumlah fenolik yang terendah sebanyak 1.46 ± 0.29 % d.w. TAE, manakala, Myrica javanica mengandungi jumlah fenolik yang tertinggi sebanyak 4.84 ± 0.32 % d.w. TAE. Spesies Podocarpaceae menunjukkan kandungan fenolik yang tinggi kecuali Dacrycarpus imbricatus. Korelasi antara kandungan fenolik dengan kandungan nitrogen adalah negatif (r = - 0.8030 and  $R^2$  = 0.6449) dan signifikan (p<0.01). Nisbah C:N dalam daun pokok adalah signifikan (p<0.05). Korelasi antara nisbah C:N dengan kandungan nitrogen dalam daun adalah negatif (r = - 0.9600 and  $R^2$  = 0.9224) dan signifikan (p<0.01). Nisbah C:N menunjukkan kualiti daun dan keupayaan pereputan sarap daun. Nisbah C:N yang tinggi menunjukkan kualiti sarap daun yang rendah dan mengurangkan keupayaan pereputan sarap daun. Kualiti sarap daun menentukan kadar pereputan kerana kualiti sarap daun berubah akibat daripada penggunaan karbon dan nitrogen oleh pengurai. Kualiti sarap daun yang rendah dapat mengakibatkan kekurangan nitrogen, seterusnya mengakibatkan peningkatan kandungan fenolik dalam tumbuhan. Tumbuhan yang menghadapi masalah kekurangan nutrien mengalami kadar tumbesaran yang rendah serta mengandungi sebatian metabolit sekunder yang berkarbon tinggi. Korelasi negatif antara fenolik dengan nitrogen dalam daun adalah seiring dengan hipotesis "carbon-nutrient balance" bahawa ketersediaan nutrien dalam tanah mempengaruhi sintesis sebatian metabolit sekunder. Tumbuhan yang menghasilkan kandungan fenolik yang tinggi mungkin mengamalkan strategi tertentu supaya dapat beradaptasi dalam persekitaran yang kekurangan nutren.



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## LIST OF SYMBOLS AND ABBERATIONS

- °C Degree Celsius
- % Percentage
- ha Hectare
- m Meter
- ml Milliliter
- cm Centimeter
- mm Milimeter
- nm Nanometer
- M Molarity
- g Gram
- µg microgram
- µl Microliter
- I Liter
- mg/l Milligram per liter
- w/w Percentage weight per weight
- % d.w. Percent dry weight
- ppm Parts per million
- rpm Revolutions per minutes
- m asl meter above sea level
- N Nitrogen
- N<sub>2</sub> Atmospheric nitrogen
- OH Hydroxyl group
- NH4<sup>+</sup> Ammonium
- NO<sub>3</sub> Nitrate
- C Carbon
- NO<sub>2</sub> Nitrite
- NH<sub>3</sub> Ammonia



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#### **CHAPTER 1**

#### INTRODUCTION

#### **1.1** An overview

Plant secondary metabolites are natural chemical product in plants that served no functions and effects in its primary metabolic processes such as growth, fecundity and development. Basically these secondary metabolites are the end products of plants primary metabolism, and widespread in high plants species occuring particularly large amount in plant tissue. They play vital roles in the adaptation of plants to their environment and perform in plant fitness by interacting to their environment. As reviewed by Wink (2003) pattern of secondary metabolites present in a given plant is often complex due to its ability to change plant tissue and organ in a specific way and difference are relatively conspicuous between different developmental stages, between individual and populations.

Polyphenols are polymers of phenols, compound that consists of a hydroxyl substituent bonded with an aromatic ring. Polyphenols can be further categorized into lower molecular weight phenolic compound such as simple phenols, flavonoids and high molecular weight phenolic compound such as tannins and lignin (Hattenschwiler & Vitousek, 2000). Distribution and accumulation of polyphenols in plants varies from species to species. Polyphenols may occur in leaves, buds, barks and roots (Kraus *et al.*, 2003). Plant derived polyphenols are relatively versatile in many ways where it is not only beneficial to plants itself, but to human as well as to the ecosystem.



Polyphenol could affect the composition and activity of decomposer community, thus leading to influence the rate of decomposition and nutrient cycling (Hättenschwiler & Vitousek, 2000). On top of that, polyphenol also alter nitrogen availability by forming protein-polyphenol complexes (PPC) when polyphenol binds with protein molecule through hydrogen bonding and hydrophobic interaction (Kraus *et al.*, 2003). PPC served as important factor in controlling nitrogen dynamic probably due to its resistant to degradation that leads to limit nitrogen mineralization (Majuakim & Kitayama, 2013). Polyphenol also affect nitrogen mineralization by increase foliar carbon to nitrogen (C:N) ratio and leaf toughness (Majuakim & Kitayama, 2013).

Nitrogen is a macronutrient essential to the well-being of plants, primarily for plant growth. Plants can absorb nitrogen in both organic and inorganic form. Leaf nitrogen is vital for plants photosynthesis. Strong relationship between leaf nitrogen and photosynthetic activity has been known (Drouet & Bonhomme, 1999) primarily because nitrogen in leaf comprises proportional amount of chlorophyll and major photosynthetic enzyme. Thus, change in leaf nitrogen content might induced changes to the thylakoid pigment protein complex, thereby, directly affect the physiological functioning of plants.

On top of that, accumulation and biosynthsis of polyphenols such as phenolic compounds are common response to abiotic and biotic stress (Juszczuk *et al.*, 2004). Biosynthesis of polyphenols in leaves also depend on the nutrient status of the soil, leading to greater production of polyphenol content in soil deficient in nitrogen and phosphorus (Kuiters & Denneman, 1987). Nevertheless, phenolic content in plant leaves increased in response to nitrogen limitation (Løvdal *et al.*, 2010). Polyphenols are made up of carbon frameworks, increasing polyphenols in leaves leading to increase in total carbon content in leaves in response to nitrogen depletion. Thereby, production of phenolic may in turns participate in the process of stress acclimation. Therefore, in an environment where nitrogen availability is low, resulting in considerably high amount of phenolic synthesized in leaves in order to compensate their nutrient limitation.



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## 1.2 Justification

Polyphenol has an implication in altering nitrogen dynamics. Soil-polyphenol interactions have been continuously investigated in previous studies. However, research on investigating the relationship between nitrogen and polyphenol in leaves is negligible. Nitrogen content in leaf is important because photosynthetic capacity of leaves is related to their nitrogen content. Also, the proteins of the Calvin cycle and thylakoids represent the greatest fraction of leaf nitrogen content. This study may provide the link between nitrogen and polyphenols in leaves as well as the effects of nitrogen to polyphenols in leaves.

## 1.3 Objectives

- 1) To determine whether there is correlation between nitrogen and phenolic compounds in tree leaves.
- 2) To compare nitrogen and phenolic compounds in leaves of selected tree species.

## 1.4 Hypothesis

 $H_0$ : No correlation relationship between N and phenolic compounds in tree foliar and there is no different in concentration of N, and total phenolic between tree species.

H<sub>1</sub>: A correlation relationship (positive or negative) between N and phenolic compounds in tree foliar and there is different in concentration of N, and total phenolic between tree species.



#### **CHAPTER 2**

#### LITERATURE REVIEW

#### 2.1 Secondary metabolites in plants

Plants share with all other living organisms a number of biochemical reactions that maintain their basic or primary metabolism, which is involved in the formation and breakdown of a limited set of chemicals. Based on the primary metabolism, plants have evolved a corona of secondary metabolic pathways producing an array of secondary plant substances, namely plant secondary metabolites. The large variety of secondary constituents is produced via biogenetic pathways, each leading to one or a few key metabolites, from which numerous derivatives are formed, usually by a consecutive series of enzymatic transformations (Hartman, 1996; Haslam, 1998; Cseke, 2006).

Secondary plant metabolites are natural chemical products in plants that serve less important functions in plants. According to Bourgaud *et al.*, (2001) plant secondary compounds were indicated by their low abundance, often composed not more than 1% of the total carbon. Thus, the great majority of which do not appear to participate directly in growth and development of plants. In contrast, primary plant metabolites, such as phytosterols, acyl lipids, nucleotides, amino acids, carbohydrates and organic acids, were all found in plants. These compounds performed important metabolic roles in basic life functions of the plant, such as, cellular division, respirations, storage, fecundity, growth and development, their importance were usually evident. Primary plant metabolites were as well involved in fundamental plant physiological processes that forming essential nutrient for herbivores. Berenbaum (1995) explained that qualitative and quantitative variation in primary plant compounds can have profound effects on insect preference and performances.

Wink (2003) reported that secondary plant metabolites are widely distributed in the plant. To date, though several thousands of secondary metabolites have been identified (Schwab, 2003), nevertheless, only few biosynthetic pathways of these secondary compounds have been fully elucidated (Schoonhoven *et al.*, 2005). Often, plant secondary metabolites were classified according to their biosynthetic pathways (Bourgard *et al.*, 2001; Agostini-Costa *et al.*, 2012). The three main groups classified in plant secondary metabolites were terpenes, phenolics and alkaloids. The major secondary compounds were often accompanied by several derivatives and minor components.

As such, the complexity of chemical structure in secondary metabolites in a given plants might induced regular changes in plant tissues and organ specific ways; differences can be seen between different developmental stages (Wink, 2003). Functioning of plant secondary metabolites are specific to the plants in which they are found in (Tanaka *et al.*, 1999). Particularly the most common investigated families of polyphenol are phenolics due to its involvement in lignin synthesis which is common in higher plants (Bourgaud *et al.*, 2001). In addition, derivatives of flavonoids (belongs to phenolic group), the anthocyanins, provide the red and red blue pigments of flower petals with abundances of approximately 30 % of the dry weight of some flower (Williams & Grayer, 2004). Polymerization of catechin and leucoanthocynidins yields condensed tannin which exhibits the color of tea (Cseke *et al.*, 2006).

Besides that, secondary plant metabolites play vital roles in the adaptation of plants to their environment and performance in plant fitness by interacting with the environment. Therefore, lacking secondary plant metabolites does not caused sudden death but rather in long term impairment of plant's survivability (Agostini-Costa *et al.*,



2012). Secondary plant metabolites have important biological and pharmacological activities such as antibiotic, antifungal, antiviral, which functions to protect plants from pathogen attacks as well as anti-germinative properties (Bourgard *et al.*, 2001, Stankovic, 2010). Anti-feeding properties have also been reported for the prevention of herbivore for building plant defense mechanism (Bourgard *et al.*, 2001; Wink, 2003; Schoonhoven *et al.*, 2005; Agostini-Costa *et al.*, 2012). Because of its biological and pharmacological activities, the productions of high value secondary metabolites are of plant origin. Nowadays, secondary metabolites correspond to valuable compounds such as pharmaceutics, cosmetics, fine chemicals, food addictives, flavours, fragrances and colors. For instance, in pharmaceutical industry aspirin (acetylsalicylate) had been derived from salicylate (Wink, 2003). Production of pungent food addictives, capsaicin, natural color anthocyanin and natural flavor had also been derived from natural plant (Rao & Ravishankar, 2002).

## 2.2 The chemistry of phenolic compounds

Phenolic compounds were widely distributed in nature and they were ubiquitous in plants. Vast majority of plant-based aromatic secondary metabolites consist of phenol. Phenolic compounds were organic compounds that posses one or more hydroxyl (OH) substituents bonded to an aromatic ring; normally derived from the simple parent substance, phenol (Waterman & Mole, 1994). Polyphenol was formed when the aromatic ring was bonded with several or many phenolic hydroxyl sunstituents. However, not all hydroxyl groups are phenolic, in the case when hydroxyl groups bonded to a non-cyclic structure or to a non-aromatic cyclic which serve no properties of phenol. Phenolic compounds are products of secondary metabolism of plants and arise biogenetically from two main primary synthetic pathways: the shikimate pathway and the acteate pathway (Paixao *et al.*, 2007).

Numerous categories of phenolic included simple phenol, phenylpropanoids, flavonoids, tannin, and quinones (Cseke *et al.*, 2006). Polyphenol could be further categorized into two subcategories: low molecular weight phenolic (LMP) and high



molecular weight phenolic (HMP). Phenolic compounds have various types of chemical structures which enable easy differentiation. Chemical structures differ in low molecular weight phenolic or simple phenols, such as hydrobenzoic acid derivatices and catechols, as well as long chain polymers in high molecular weight phenolic, such as catechol melanin, lignin, and tannin (Agostini-Costa *et al.*, 2012).

Tannin are polyphenolic compounds with molecular weight ranges between 500 to 3000 daltons, where they could be easily found in all classes of vascular plants, often in high concentration (Haslam, 1998; Hättenschwiler & Vitousek, 2000; Kraus *et al.*, 2003; Majuakim & Kitayama 2013). Tannin is water soluble phenolic compounds with the ability to precipitate alkaloids, gelatin and other proteins (Haslam, 1998). Tannin is commonly classified into two types: the hydrolysable tannin and the non-hydrolysable, condensed tannin. Tannin also present in vegetal products as complex mixtures in which predominates esters of some polyphenolic acids with glucids, in the case of hydrolysable tannin, or products or products of catechin condensation, which able to give steady, characteristic bounds, with the amino acids from proteins structure.

Flavan-3-ol or catechin is the fundamental structure units of condensed tannin (Haslam, 2007). Condensed tannin, a type of structural-complex tannin, can exist in the form of oligomers or polymers, where both forms are made up of two to ten or more catechin units (Haslam, 2007). The more catechin units binding together, the more complex the structure of the tannin is. Thus, calculation of the number of hydroxyl groups attached to the B-ring of tannin enabled us to distinguish the types of tannin found. For instance, procyanidins have a di-hydroxy B-ring while prodelphinidins have a tri-hydroxy B-ring (Kraus *et al.*, 2003). Procyanidins and prodelphinidins are commonly found in certain types of condensed tannin present in plants that possess a woody habit of growth (Haslam, 1998). On the other hand, hydrolysable tannin are esters of a sugar (usually glucose molecule) with one or more trihydroxybenzenecarboxylic acid or gallic acid (Cseke *et al.*, 2003).



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There are significant differences between polyphenols from the variation in chain length, number of hydroxyl groups, positions of intermonomer linkages, stereochemistry, branching extent, glycosylation and also in its substitutions with aliphatic or carbohydrates moisteis (Kraus *et al.*, 2003, Kraus *et al.*, 2004). Because of these variations, certain polyphenols may exist only in certain plants . For instance, gymnopserms and monocotyledonous only synthesize condensed tannin, while dicotyledonous can produce both condensed tannin and hydrolysable tannin or a mixture or both (Haslam, 1998; Kraus *et al.*, 2003). Since there is great diversity of tannin presence in different plants, indicating the greater probability of variation may occur (Behrens *et al.*, 2003 ; Kraus *et al.*, 2003). As a result, plants are able to produce large variety of tannin, and each tannin compounds in every individual plant would have its own distinctive roles (Hagerman, 1998b).

## 2.3 Polyphenols in plants

## 2.3.1 Diversity and distribution of polyphenols in plants

Plants are able to synthesis thousand of phenolic and polyphenolic compounds such as phenolic acids, tannin, flavonoids and lignin as secondary metabolite. Diversification in structure of plant polyphenols are relatively essential to the physiology of plants such as being involved in lignifications and structure, pigmentation, pollination, allelopathy, pathogen and predator resistance, and growth (Haslam, 1998). The majority are synthesized by the highly-branched phenylpropanoid pathway, which is responsible for the biogenesis of a large number of compounds of considerable structure diversity (Duthie *et al.*, 2003).

Considerable accumulation of polyphenols concentration are determined less frequently in plant parts other than leaves and not much research has been reported in fine roots (Hättenschwiler & Vitousek, 2000). Bending & Read (1996) reported that greater amount and greater diversity of tannin were formed within leaves. Generally, level of tannin found in most plant tissue was in the range of 2 - 5 % of its fresh weight (Haslam, 2007). However, leaves of sumach (*Phus typhina*) were reported to



contain tannin concentration as high as 12 % of its dry weight (Haslam, 2007). Ossipov *et al.*, (2001) reported that mountain brich leaves (*Betula pubescens ssp. czerepanovli*) have significantly high level of phenolics which often exceeds 10% of its dry mass.

Despite of its distribution in leaves, fruits, buds, bark and roots, polyphenols spread through the epiderm, hypoderm, periderm, mesophyll, vascular tissue and companion cells, of which its distribution is relatively different from species to species (Kraus *et al.*, 2003). Tannin is found as smaller molecules within vegetal organs with intense physiological activity (Palici *et al.*, 2005). In addition, macromolecule tannin are accumulated in ligneous tissues, barks, and roots of plants. Tannin were also reported to be stored in vacuoles, bound to cell wall components or even forming complexes with protein within the plant. The complexes are believed to be formed during cellular degradation (Kraus *et al.*, 2003). Besides that, tannin also occurred as soluble components in the sap of living cells (Schoonhoven *et al.*, 2005), particularly parenchymatic cells as cortical parenchyma, secondary xylem and pith, or in specialized cells like idioblastes (Palici *et al.*, 2005).

According to Nierop & Filley (2007) tannin and lignin were found to be the richest, most abundant plant biopolymers existing in nature after cellulose and hemicelluloses. Content of tannin might be relatively lesser than lignin, but tannin content in leaves may exceed lignin amount in soft tissue of tree foliar as well as needles. Maie *et al.*, (2003) reported that compared to hydrolysable tannin, the amount of condensed tannin in woody plants is relatively more abundant and are more insoluble. Swain (1979) reported that hydrolysable tannin are limited in angiosperms, while condensed tannin, on the other hand, are widespread in the plant kingdom.



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