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Faculty of Tropical AgriSciences

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Underutilized edible fruit tree species of the Philippines as potential sources of antioxidants

MASTER´S THESIS

Prague 2019

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Declaration

"I, Jana Tulková, hereby declare that this thesis entitled "Underutilized edible fruit tree species of the Philippines as potential sources of antioxidants" submitted in partial fulfilment of the requirements for the degree of Master of Science at the Faculty of Tropical AgriSciences of the Czech University of Life Sciences Prague is my own independent work unless otherwise referenced or acknowledged.

April 24, 2019, Prague

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Acknowledgement

I would like to express my sincere gratitude to my thesis supervisor Ing. Johana Rondevaldova Ph.D. (Department of Crop Science and Agroforestry, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague) for her kind attitude, wise comments, ideas, professional guidance and patience during the process of thesis writing. I would like to also thank to Ing. Markéta Houdková Ph.D. and Ing. Marie Netopilová (both from Department of Crop Science and Agroforestry, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague) for patience, encouragement and useful advices during laboratory works.

I am also grateful to my family and loved for patience, encouragement and providing me all facilities required.

Abstract

In present, human demand for plant-based product with health promoting effect has increased. Indigenous plant species, used in traditional cuisine and medicine has been frequently tested for antioxidant activity as a prevention of oxidative stress and subsequent diseases (cancer, anaemia, Parkinson disease).

This thesis focuses on evaluation of *in vitro* antioxidant activity of underutilized fruit tree species growing and used in Philippine cuisine. For the assessment of antioxidant activity of methanol extracts, 2,2-diphenyl-1-picrylhydrazyl radical assay (DPPH), oxygen radical absorbance capacity (ORAC) assay were used and total phenolic content assay (TPC) was estimated. The strongest antioxidant activity possessed *Stelechocarpus burahol* with IC_{50} 253.7 μg/mL (DPPH), 32.2 μg/mL (ORAC) and *Broussonetia luzonica* with IC_{50} 90.9 μg/mL (DPPH), 37.9 μg/mL (ORAC). TPC was the highest at *S. burahol* with 72.2 mg/g and *B. luzonica* with GAE 133.0 mg/g.

In scientific journals was not found any previous antioxidant activity evaluation of inflorescence *B. luzonica*. The fruit of *S. burahol* also was not tested, but in comparison to other plant parts tested in scientific articles, fruits of *S. burahol* possessed the strongest activity.

S. burahol and *B. luzonica* have the potential for further practical application, assuming prior determination of their chemical composition. These species can contribute to species diversity of agricultural and forest composition and they are suitable for growing in multi-storey (*B. luzonica*) and Taungya (*S. burahol*) agroforestry systems.

Key words: antioxidant activity, DPPH, ORAC, TPC, Philippine plants, underutilized species

Abstrakt

V současné době vzrostla poptávka lidí po rostlinných produktech s účinkem podporujícím zdraví. Původní druhy rostlin používané v tradiční kuchyni a medicíně jsou často testovány na antioxidační aktivitu, jako prevenci oxidačního stresu a následných onemocnění (rakovina, anémie, Parkinsonova choroba).

Tato diplomová práce se zaměřuje na zhodnocení antioxidační aktivity, *in vitro* podužívaných druhů ovocných stromů pěstovaných a využívaných ve filipínské kuchyni. Pro stanovení antioxidační aktivity metanolových extraktů byl použit test inhibice radikálu 2,2-difenyl-1-pikrylhydrazyl (DPPH), stanovení kapacity absorpce kyslíkových radikálů (ORAC) a stanovení celkového obsahu fenolických látek (TPC). Nejsilnější antioxidační aktivita byla stanovena u druhu *Stelechocarpus burahol* s IC_{50} 253,7 μg/ml (DPPH), 32,2 μg/ml (ORAC) a *Broussonetia luzonica* s IC50 90,9 μg/ml (DPPH), 37,9 μg/ml (ORAC). Obsah fenolických látek byl nejvyšší u druhu *S. burahol* s 72,2 mg/g a *B. luzonica* s GAE 133,0 mg/g.

Ve vědecké literatuře nebylo nalezeno předchozí testování květenství druhu *B. luzonica.* Plody druhu *S. burahol* také nebyly testovány, ale ve srovnání s jinými rostlinnými částmi testovanými ve vědeckých článcích, vykazovaly plody druhu *S. burahol* nejsilnější aktivitu.

S. burahol a *B. luzonica* mají potenciál pro další praktickému využití, za předpokladu stanovení jejich chemického složení. Tyto druhy mohou přispět k diverzitě druhů používaných pro zemědělské a lesnické účely a jsou vhodné pro pěstování ve víceúrovňových agrolesnických systémech (*B. luzonica*) a systémech Taungya (*S. burahol*).

Klíčová slova: Antioxidační aktivita, DPPH, ORAC, TPC, Filipínské rostliny, nevyužívané rostliny

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AAPH	2,2′-azobis(2-amidino-propane) dihydrochloride
ABAP	2,2′-diazobis-(2-amidinopropane)dihydrochloride
ABTS	2,2′-azinobis(3-ethyl-benzothiazolline-6-sulfonic acid
ASEAN	Association of Southeast Asian Nations
CAT	Catalases
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl Radical
FRAP	Ferric Ion Reducing Antioxidant Power Assay
GAE	Gallic acid equivalent
GDP	Gross Domestic Product
GPx	Glutathione peroxidase
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
IC_{50}	Half maximal inhibitory concentration
OH	Hydroxyl radical
$ONOO^-$	Peroxyl radical
ORAC	Oxygen Radical Absorbance Capacity
O_2	Superoxide anion
NBT	Nitrogen Blue Tetrazolium
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
ROO	Peroxynitrite
R-PE	R-phycoerythrin
SOD	Superoxide dismutase

TEAC	Trolox Equivalent Antioxidant Capacity Assay
TPTZ	2,4,6-tri(2-pyridyl)- 1,3,5-triazine
TRAP	Total Peroxyl Radical-Trapping Antioxidant Assay
TPC	Total phenolic content Assay
UV-Vis	Ultraviolet – visible spectroscopy

1 Introduction

Historically, positive influence of some fruits, vegetables and herbs to human wellbeing has been known. The sailors at the long sailing ship voyage suffered by scurvy, the disease characterized by weakness, bloody spots and loosing teeth, ending with the death. The illness was treated in few days at the land just by eating fresh acidic fruit (Carpenter, 2004). In past, the reason why it happens and what causes the healing was not clear. However, clarification began with identification of vitamins A, C and E as health promoting agents and subsequently antioxidants were revolutionized.

The human hunt for mitigation of aging, cancer prevention and treatment has been widely expanded across entire world. Amazing indigenous medicine and cuisine habits are scientifically tested for various health promoting and life prolonging effects. The tropical and subtropical areas of the world provide wide range of plants with high content of beneficial substances.

In present, the fruits presented by the term “super food” or “super fruit” have been popularized by trade companies. Many indigenous plants from the Amazon rainforest, Tropical Africa and South East Asia became very lucrative. For example Açaí berry (*Euterpe oleracea*), originating in Brazilian rainforest, is a fruit with high antioxidant activity, high in fibre and with omega fatty acids (Kang et al., 2012). This fruit is nowadays well traded especially in Western Europe and American continent as a fresh fruit or health promoting supplement. Also South-East Asian Gac fruit (*Momordica cochinensis*) is for its high content of carotenoids, polyphenols, vitamin C and E, called as age-defying “superfruit” (Kubola and Siriamornpun, 2011).

Even in present, some species in wild nature have not been tested for antioxidant activity. Some of these are already used for treatment of various illnesses by local people or just eaten as local fruit, so possibly neglected and underutilised.

This thesis focuses on tree species from the Philippines, already used in local cuisine, with possible antioxidant activity, what could reveal added value of the species in human nutrition and also enrich the composition of the tree species used in agroforestry systems.

2 Literature Review

2.1 Free radicals

The free radical is explained as any species of independent existence with one or more unpaired electrons. It may be superoxide (O_2), thiyl (R-S) or nitric oxide (NO). Free radical could be produced by biological combustion, respiration and cell-mediated immune functions. Also environmental conditions generate harmful free radicals, for example cigarette smoke, automobile exhaust fumes, radiation, air pollution and pesticides. They lead to cumulate free radicals and cause damage of lipids, DNA, proteins, carbohydrates, membranes, resulting oxidative stress (Tiwari *et al*. 2001).

MacDonald-Wicks *et al.* (2006) describes six mayor reactive oxygen species (ROS) and reactive nitrogen species (RNS) species in human organism and food-related systems: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radical (ROO), hydroxyl radical (OH), singlet oxygen (O_2), peroxynitrite ($ONOO^-$).

2.2 Antioxidants

Pullaiah (2013) explains antioxidants as a type of complex compounds found in our diet that act as a protective shield for our body against certain disastrous enemies (diseases) such as arterial and cardiac diseases, arthritis, cataracts and also premature ageing along with several chronical diseases. Whereas Smirnoff (2005) says, that antioxidants delay or prevent oxidation of organism.

To protect human cells and organs of the body against ROS, highly sophisticated system of protection, both endogenous and exogenous have evolved (Parvaiz *et al.* 2010). Table 1. presents distribution of antioxidants in human body. Only enzymatic and dietary antioxidants, closely related to this thesis, were described further.

Table 1. Distribution of antioxidants in human body

Endogenous Antioxidants	Nonenzymatic	Bilirubin Thiosols NADPH and NADH Ubiquinone (coenzyme Q10) Uric acid
	Enzymes	superoxide dismutase catalase glutathione peroxidase
Dietary Antioxidants		Vitamin C Vitamin E Carotenoids Polyphenols
Metal binding Proteins	Copper binding	Albumin Ceruloplasmin Metallothionein
	Iron binding	Ferritin Myoglobin Transferrin

2.2.1. Enzymatic Antioxidants

Superoxide dismutase (SOD) – This enzyme converts O_2^- to H_2O_2. SOD occurs in all aerobic organisms. The highest concentration of SOD in human body is located in liver, cerebral cortex, testicles, heart muscle and kidneys (Racek, 2003).

Catalases (CAT) – The enzyme CAT is responsible for the degradation of H_2O_2 to oxygen and water. It is present in all animal cells. The highest content of enzyme Catalases is in erythrocytes and peroxisomes of liver cells (Racek, 2003, Štípek *et al.*, 2000)

$$2H2O2 \longrightarrow 2H2O + O2$$

Glutathione peroxidase (GPx) – The enzyme GPx is responsible for protection of cell from damage due to free radicals of lipid peroxides and hydrogen (Racek, 2003).

Glutathione reductase (GR) – It protects cells against oxidation and contributes to regeneration of vitamin E by reduction of vitamin C and E radicals (Racek, 2003).

2.2.2. Dietary Antioxidants

Ascorbic acid (Vit. C) – Ascorbic acid reduces Fe(III) to Fe(II) and Cu (II) to Cu(I). Vitamin C is very active antioxidant and reduces both organic and inorganic radicals, such as O_2, HO_2, HO, hydrophilic RO_2 and NO_2. It is capable to neutralizing ROS in aqueous phase, before lipid peroxidation is initiated (Štípek et al. 2000; Krishnamurthy and Wadhwani, 2012).

α – Tocopherol (Vit. E) – Vitamin E consists of eight isomers of which α-tocopherol is the most effective. The α-tocopherol is a lipid soluble and the most effective chain-breaking antioxidant of the cell membrane by protecting the membrane fatty acids against lipid peroxidation. When lipids peroxidase, α-tocopherol converts alkyl peroxyl radical (LOO) to H_2O_2 (Štípek et al. 2000; Krishnamurthy and Wadhwani, 2012).

Carotenoids – Carotenoids are isoprene compounds, belonging to terpenes and divides in groups according to number of cyclohexenyl rings. The α and β carotenes with two rings, γ and δ with one ring and lycopene as linear carbohydrate with no ring. β-carotene is important precursor for vitamin A (retinol), which significantly affects mechanism of vision (Štípek et al., 2000).

Phenolic compounds – Phenolic compounds are natural metabolites, distributed in plants as an antimicrobial protection. In human body, phenolic compounds play and an important role in defence against environmental stresses.

They are divided in four groups: phenolic acids, flavonoids, stilbenes and tannins. Phenolic acids, with main source in tea, berries, coffee and fruit juices, consists two subgroups, the hydroxybenzoic acid (gallic acid, vanilic acid) and hydroxycinnamic acid (caffeic acid, ferulic acid and *p*-coumaric acid). Flavanoids as a most abundant group are contained in dark fruits (blackberries, plum) and have low molecular weight. Tannins are water-soluble polyphenols and they are found, for example in grape, walnut and apple juice. Stilbenes are mostly in soy and peanut products (Ozcan *et al.* 2014).

2.3 Human health and oxidative stress

Free radicals do not occur only inside of the body. Free radicals in food contribute to several damages of organism, such as destruction of vitamins, damage of pigment (Hoffmann, 2003), DNA mutation and protein damage (Cai *et al.*, 2004). This phenomenon is oxidative stress, which brings imbalance between presence of ROS/RNS and capacity of organism to fight their action by the antioxidative protection systems (Persson *et al.*, 2014).

The oxidative stress leads to many illnesses, e.g. degenerative disease, anaemia, cancer, cardiovascular disease (Cai *et al.*, 2004), diabetes (Ullah *et al.*, 2015), Parkinson disease (Sarrafchi *et al.*, 2016) and others. Many investigations reveal that phenolic and flavonoid compounds, occurring in plant tissues, could have significant potential to affect ROS and to prevent oxidative stress in human body (Gill *et al.*, 2010).

2.4 Antioxidant activity

MacDonald-Wicks *et al.* (2006) explains antioxidant activity as the rate constant of a reaction between a specific antioxidant and oxidant, whereas antioxidant capacity is a measure of the amount in moles of a given free radical scavenged by a sample. For assessment of antioxidant activity have been developed methods focused on ability to be a hydrogen donor or electron donor. The most widely used assays to screen antioxidant activity *in vitro* are described below:

2.4.1. Hydrogen atom transfer assays

ORAC – Oxygen radical absorbance capacity

This method was invented by Ou *et al.* (2001) and evaluates antioxidant activity of biological samples *in vitro*. The assay measures the oxidative degradation of fluorescein after being mixed with free radical 2,2´-azobis(2-amidino-propane) dihydrochloride (APPH). APPH produces the $ONOO^-$, which damages fluorescein molecules and results in loss of fluorescein. Trolox (6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid) the water-soluable analog of vitamin E, is used as a

reference compound, because it is vitamin with proven antioxidant activity and it reacts against recognised ROS.

TRAP – Total Peroxyl-trapping Antioxidant Parameter Assay

The TRAP assay was developed to measure the total antioxidant status of human plasma. The luminescence spectrometer is used to measure the fluorescence decay of R-phycoerythrin (R-PE) during peroxidation reaction, produced in controlled rate. For initiation of the reaction could be used, for example 2,2′-diazobis(2-amidinopropane)dihydrochloride (ABAP). The TRAP values are calculated as the length of the lag-phase caused by the antioxidant compared to Trolox (MacDonald-Wicks *et al.,* 2006; Moharram and Youssef, 2014).

2.4.2. Electron transfer assays

DPPH – 2,2-Diphenyl-1-Picrylhydrazyl Radical scavenging: spectrometric assay

The method was developed by Sharma and Bhat (2009). It is useful for evaluation of antioxidant activity in rapid and cheap way. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay radical scavenging test is technically simple and needs only UV-Vis spectrophotometer to perform, at maximum wavelength 515 nm (He *et al.,* 2012). In presence of free radical-scavenging antioxidants, hydrogen donor receives proton, absorption intensity decreases and dark purple DPPH radical solution loses its chromophore and changes to yellow (Zamora *et al.,* 2012)

TEAC – Trolox Equivalent Antioxidant Capacity

The TEAC assay was developed for testing of antioxidant capacity in food samples. The assay is similar to ORAC and measures the loss of colour, when an antioxidant is added to blue-green 2,2′-azinobis(3-ethyl-benzothiazolline-6-sulfonic acid ($ABTS^+$). Antioxidant reduces blue $ABTS^+$ radical to ABTS and decolorizes the solution. The extent of decolourisation of $ABTS^+$ in percentage inhibition is determined as function of concentration and time and calculated relative to standard Trolox.

The TEAC assay has a great disadvantage in $ABTS^+$, which is not found in biological systems and the radical is not even similar to some occurring in those

systems. Otherwise, the advantage of this assay is in the point, that the assay could be used in aqueous and lipid phase and thus the antioxidant activity could be determined in both (MacDonald-Wicks *et al.*, 2006; Moharram and Youssef, 2014).

FRAP – Ferric ion reducing antioxidant power assay

The FRAP assay was originally designed as assay for determination of antioxidant activity of plasma. The assay measures the ability of antioxidant to reduce the ferric ion 2,4,6-tri(2-pyridyl)- 1,3,5-triazine (TPTZ), which is used as an oxidant. The Trolox or Ascorbic acid is used as reference. The reduction is monitored at diode-array spectrophotometer, by measuring the change in absorption at 593 nm.

There is just a little difference between TEAC assay and FRAP assay. The redox potential of Fe (III) salt is similar to $ABTS^{-}$ with difference in pH. The TEAC assay is performed with neutral pH, whereas FRAP need acidic condition (pH3.6) (MacDonald-Wicks *et al.*, 2006; Pisoschi and Negulescu, 2011; Moharram and Youssef, 2014).

TPC – Total Phenol Content Assay by Folin-Ciocalteu Reagent

The Total phenol content method by F-C is simple method belonging to electron transfer group, which determines only content of phenolic compounds. However, phenolic compounds are often responsible for antioxidant activity of the sample.

The method measures the reducing capacity of the sample. The phenolic compounds react with FC in basic conditions, where separation of the phenolic proton leads to a phenolate anion, which is able to reduce F-C. The product of metal oxides reduction, blue in colour, exhibits a broad absorption at maximum 765 nm. The intensity of absorption is proportional to the concentration of phenols (MacDonald-Wicks *et al.*, 2006). As a standard is used Gallic acid, belonging to group of phenolic acids. Total phenolic content is estimated as Gallic acid equivalent per gram of extract (He *et al.*, 2012).

2.4.3. ROS/RNS scavenging assays

Superoxide (O_2^-) scavenging - The superoxide radical is easily formed by radiolysis of water in presence of oxygen and formate, which allows reaction rate constants to be measured. The reaction measures the ability of the superoxide to reduce nitro blue tetrazolium (NTB) or cytochrome c (for microplate format) to formazan. The formation of formazan is further measured by spectrophotometer at 560 nm (Quick *et al.,* 2000; MacDonalds-Wicks *et al.* 2006).

Hydrogen peroxide (H_2O_2) scavenging – The assay of Mueller (2000), describes a non-enzymatic hydrogen peroxide assay, based on the oxidation of luminal by sodium hypochlorite (NaOCl) to diazaquinone, which is converted by H_2O_2 to aminophtalate. The luminescence signal of this assay is 2 seconds at a minimum wavelength of 431 nm.

Peroxynitrite ($ONOO^-$) scavenging – Peroxynitrate is not strongly oxidising agent. It could be converted to peroxynitrous acid (ONOOH) at a physiological pH, which decays rapidly to a series of strong oxidising agents. The damage is mainly made by nitration or hydroxylation of aromatic compounds (MacDonald-Wicks *et al.,* 2006).

2.4.4. Plants and their antioxidant activity

Recently, there has been increase of interest in fruit and vegetables, high in health promoting compounds, commercially called “superfoods” and their food supplements. Well-known and traditionally used natural antioxidants such as wild berries, cocoa, coffee, tea, dark green vegetables, beans and spices, became important and commercially exploited goods. With high demand for natural antioxidants, many investigations of indigenous fruits and vegetables were done.

One of the species originally found only in local cuisine and recently popular species, high in health promoting compounds, is Amla (*Phyllanthus emblica*), also called Indian gooseberry. Amla is an important component for plant medicine of Ajurveda. In ancient India it was believed that Amla could impart immortality. Recently it has been identify as a species high in antioxidants (Carlsen *et al.,* 2010), with immunomodulatory and antistress activity, because of the high content of ascorbic acid,

chebulinic acid, gallic acid and corilagin in fruits (Singh *et al*., 2018). The indigenous Southeast Asian Gac (*Momordica cochinchinensis*) became famous just a few years ago. This deep orange tropical fruit is the major source of lycopene and carotenoids as antioxidant substance (Tinrat *et al*., 2014) and it has been studied as species associated with reduce risk of prostate and lung cancers (Aoki *et al*., 2002).

Whereas above mentioned species are commercially exploited, some of less known originating or introduced to the Philippines exhibit significant antioxidant activity and prevention against numerous disease. For example Jamaica cherry (*Muntingia calabura*) is species neglected in many countries. In the Philippines, the flowers are used to treat headache, cold or as a tranquillizers, antispasmodics and antidyspeptic (Mahmood *et al.,* 2014). Moreover, Jamaican cherries were also determined as an important source of phenolic compounds (Recuenco *et al.,* 2016; Rotta *et al.,* 2017). Mabolo or Velvet apple (*Diospyros blancoi*) is species traditionally used in the Philippine home medicine. The bark is used as a treatment for coughs, fevers, and dysentery. The leaves are used in snakebite and oil expressed seeds treat diarrhoea (Tropical Plant Database). However, the study of Howlader *et al.* (2012) revealed high antioxidant activity of fruits and high content of phenolic compounds (Recuenco *et al.,* 2016), which brings the added value in the Philippine nutrition.

2.5 Geographical area of the Philippines

Republic of the Philippines is island country located in Western Pacific, consisting of 7.100 islets (Hernandes *et al.,* 2019). Capital city Manila lays on the biggest island Luzon in National Capital Region, Metro Manila.

The Philippines takes its name from Spanish king Philip II., who reigned during Spanish colonization in 16th century. The country was ruled by Spanish for 333 years and colonized for 48 years by Americans, until the 4th July 1946, when the Philippines became independent. Due to historical circumstances, official language of the Philippines is English and it is one of just two Asian countries with Roman Catholic as major religion.

2.5.1. Land distribution

The Philippine archipelago is surrounded by South China Sea to the west and north, Philippine Sea to the east, Celebes Sea to south and Sulu Sea to southwest. The islands are composed primarily by volcanic rock and coral in coastal areas. About 50 volcanos occur in the Philippines, but only 10 are currently active.

The Philippine islands have very diverse topography with high mountains surrounded by narrow flat lowlands, valleys, river systems and lakes. The mountain ranges of most of the islands tend to cross the land in the same direction, run from north to south. The Cordillera Central mountain range runs across entire Luzon, forming three parallel mountain ranges with an average elevation of about 1,800 m above sea level. Even if it is highest and largest mountain range of the Philippines, the highest peak Mount Apo with 2,954 m above sea level is located at Mindanao island (Hernandes *et al.*, 2019).

Water system of the Philippines plays important role in land topography. Rivers Cagayan, Agno, Pampanga, Pasig and Bicol are the largest rivers and supply of fresh water not only human settlements but also wide rice plains and small fields.

2.5.2. Climate and Soils

The climate of the Philippines is monsoonal with periodical changing of wet and dry season. Southwest rain-bearing winds blows from May to October, whereas drier winds blows from northeast from November to February. Temperatures remain stable during the year with an average temperature of 27 ^{o}C. Whole country is located in tropical climate zone with high precipitation (average in lowlands 2030 mm) and humidity about 77 % (AsianInfo, 2010).

From June to December, tropical cyclones, called typhoons, often occur on the southeast of the Philippines. These heavy storms bring floods or high winds and may cause many deaths.

Due to volcanic origin of islands, soil conditions are very supportive for growth and cultivation of diverse plant species and agricultural crops. Dark cracking clays form

alluvial plains and terraces are suitable for rice cultivation. Volcanic ash works as soil fertilizer in fruit plantations and gardens, whereas vegetable and oil palms are planted at peat-like areas (Hernandes *et al.*, 2019).

2.5.3. Forestry and agriculture

Agriculture in the Philippines employs 39.8 % of labour force and contributes 20 % of GDP. The main agricultural crops are rice, sugarcane, coconut, cassava, bananas and pineapples. The most common livestock raised is broiler chicken, carabao, cattle, duck and fishery products (Bureau of Agricultural Statistics, 2004).

The country´s main exported items are coconut oil and coconut products, vegetable, fruit and prawns. The export mainly focuses on USA, Japan, Europe and ASEAN countries (members of the Association of Southeast Asian Nations).

In 1991, the agroforestry was widely applied to the Philippines as a response to increase of population and subsequent pressure to agriculture. Deforestation led to leaching of nutrients and annual yields decreased (Katayama and Luna, 1998).

In present, many agroforestry systems have evolved in the Philippines. In upland areas of the country predominant Alley cropping systems with hedgerow or double hedgerows planted along the contours and annual or perennial crop (such as coffee, fruits) alleys in between. In many cases, fodder trees or shrubs (e.g. *Desmodium rensonii, Gliricidia sepium, Flemingia congesta*) are planted instead of hedgerows and used in a cut and carry method as a fodder for animals. Another widely used agroforestry system is Multi-storey system with diverse canopy levels. The system is suitable for shade tolerant species. The most common systems are coconut-coffee-pineapple-banana mix (Cavite province); Albizia-coffee/cacao mix (Mindanao province) and many home gardens. In localities, where mature trees and plantation developed, Tree-crop grazing systems are used. Animal (e.g. cattle, carabao, goat, sheep) are allowed to graze underneath the trees (e.g. *Aleurites moluccana*), where improved forage grasses have been planted. Forest-based agroforestry system Taungya develops at newly established reforestation areas interplanted with agricultural crops.

The crops are planted until the tree canopies lower the light intensity for crops (Resource management for upland areas in Southeast Asia, 1995).

2.5.4. Nature conservation

From 2010 the Philippines are threaten by massive deforestation. According to Senate Economic Planning Office (2015), forest land decreased to 23 % (6.8 mil. hectares) of area of the Philippines. Since Ferdinand Marcos became president towards the end of his regime (1965-1986), forest cover decreased about half of was it was before. Logging companies were strongly supported by government and money for logging supported candidates during election campaigns.

Not only logging companies cause deforestation of the Philippines, also mining companies and forest fires, “kaingin” farming (slash and burn agriculture). Volcanic origin of Philippine islands plays its role in deforestation. In the past, volcanic eruption as well as typhoons destroyed huge areas of tropical rainforest. The loss of forest contributes to land erosion and huge landslides. This affects soil fertility and lowers crop yields, which forces farmers to use chemical fertilisers (Tacio, 2013). Eroded soil washes out to the rivers and creates silt in bottom. River mud threatens rich plant and animal ecosystems in coastal areas in mangrove forests.

The unique natural richness of the Philippines is immense. It belongs to 17 mega biodiverse countries in the world because of its geographical isolation. The islands contain two thirds of the world biodiversity and 70 % of plant and animal species. It is one of the most endangered Biodiversity hotspots in the world and since 1999 Subterranean River National Park Puerto-Princesa was enlisted in World Heritage site UNESCO, for its unique under surface cave system (UNESCO, 1999).

2.6 Underutilized fruit tree species of the Philippines

Economic growth depends on declining number of plant species. Thousands of edible plant species have been cultivated by humans throughout history, but modern agriculture is dominated by just three species: rice, maize and wheat and the current agriculture devotes to extensive homogenous farming landscape (NUS Community, 2018).

According to the ethnobotanic survey, there are still many countries with hundreds of crops suitable for agronomical purpose but not used. So called neglected or underutilized species (NUS) represent an immense wealth of agrobiodiversity, it contains many species highly nutritious, which can contribute to healthier diets worldwide and to fight malnutrition in many countries (IPGRI, 2002). NUS are suitable even for nature conservation of the landscape. Planting the local NUS can prevent the introduction of invasive alien species.

2.7 Characteristics of selected plant species

2.7.1. *Argusia argentea* (L.f.) Heine

English name: Tree heliotrope, Octopus bush

Local name: kapal kapal

Family: Boraginaceae

Synonyms*: Heliotropium foertherianum, Messerschmidia argentea, Tournefortia argentea*

Figure 1. ***Argusia argentea*** (Flora of Mozambique, 2014)

A. argentea is a species frequently found from Eastern Africa to Pacific and the Philippine islands. The tree grows in littoral rainforest in altitude 0-80 m and shorelines. Small size tree has softly woody branches with large oblanceolate silky tomen held in bold terminal rosettes (see Figure 1.). Trunk is soft-barked and knobby with a wide parasol canopy (Barwick, 2004). Leaves are oblanceolate to obovate, silvery green, pubescent, 10-30 cm long and 3-12 cm wide (Elevitch *et al.*, 2006). Small white flowers grow in inflorescence, which ripen to cream

brown drupe, seated on a large hairy calyx with 4 seeds per each drupe (Cooper and Cooper, 2004). This tree is salt resistant, adapted to nutrient-poor soils and limestone. It could resist to strong wind from the ocean margins with exposure to intense light (Staples and Herbst, 2005).

In New Caledonia is *A. argentea* used as treatment against consummation of exotic poisonous fish. In Fiji, the plant is used for treating of stomach troubles and in Karibati, the juice is extracted from the leaves to reduce fever. In India and the Philippines, people are using leaves for its parsley-like taste and in some areas as substitution of tobacco (Barwick, 2004).

2.7.2. *Broussonetia luzonica* (Blanco) Burr. Var. *luzonica*

English name: Birch flower

Local name: himbabao

Family: Moraceae

Broussonetia luzonica, also known as himbabao is an endemitic species of the Philippines, distributed from northern Luzon to Mindanao. It is medium-sized tree with a height up to 7 m and 60 cm in bole diameter. Leaves are alternate, rounded at the base, pointed at the apex with hairy undersurface. Small male flowers grow on the long spikey flowering branches in clusters (see Figure 2.), whereas irregularly round dark green female flowers are attached to the stem. Fruits are globose with numerous seeds (Blanco, 2017). *B. luzonica* grows in lowland thickest and second growth forest throughout the Philippines (Blanco, 2017).

Figure 2. *Broussonetia luzonica* (Pinoytrees, 2012)

Young leaves and male flowers are sold in markets and widely used cooked as a vegetable (Lafrankie, 2010). Durable bark is used for twine manufacture. Himbabao is traditionally used in Chinese medicine for healing eye disorders and impotence (Blanco, 2017).

2.7.3. *Canarium ovatum* Engler

Figure 3. ***Canarium ovatum*** (Anthropogen, 2008)

English and local name: Pili nut

Family: Burseraceae

Canarium ovatum, called pili or pili nut is native to the Philippines and introduced to Australia. Evergreen tree reaches the height of 20 m and 50 cm in diameter (Seidenschwarz, 1994). *C. ovatum* grows in low altitude of primary or secondary forest up to 500 m above sea level. Leaves are imparipinnate, spirally arranged, 40 cm long with oval leaflets. Inflorescence is small with 7 mm long male flowers and 8 mm long female flowers. Both male and female flowers grow close together at the end of the branches. Fruit drupe is ovoid, 4-7 cm long, turning from light green to purple and black (see Figure 3.) (Orwa *et al.*, 2009).

C. ovatum grows in lowland tropical rainforest with stable temperature and high precipitation. It prefers well-drained soil, either light or heavy. Mature trees can resist heavy winds. The tree is cultivated mostly by accident, as self-sown seedling in hemp and coconut plantations (Howes, 1948).

The kernel of the fruit of several species is valued nut in much of tropical Asia called pili nut (Lafrankie, 2010). Kernel contains 71,1 % fat, 11,4 % proteins and 8,4 % carbohydrates, high content of calcium, phosphorus and potassium (Orwa et al., 2009). Nuts are consumed raw or cooked and its kernel is used in chocolates, baked goods and ice cream (Kakuda *et al.* 2000). Young shoots can be eaten raw in salads and pulp oil is widely used for cooking. Resin of the tree is internationally exported as a Manila or Philippine gum elmi for purpose of ointment for healing woulds (Orwa *et al.*, 2009).

2.7.4. *Dillenia philippinensis* Rolfe.

Figure 4. ***Dillenia philippinensis*** (Phytoimages, 2006)

Local name: katmon

Family: Dilleniaceae

It is stocky endemitic tree in the Philippines. It is abundant at low elevations of dry mountainous regions up to 450 m (Pancho, 1979). The tree reaches height of 17 m and 55 cm in diameter (Brown, 1920). Leaves are subelliptic, shallow ridged, 10-20 cm long and 7-12 cm wide. Flowers grow solitary or in few clusters (see Figure 4.), with white petals, yellow stamens, purplish filaments and dark red stigmas. Fruits are globose, pale green, edible and sour in taste (Pancho, 1979). The tree prefers well-drained, moist soils. It is planted in parks and gardens for the ornamental purpose.

Fleshy fruit are used as an excellent sauce and jam or for flavouring of fish. Tree trunk provides red dye (Brown, 1920).

2.7.5. *Ficus pseudopalma* Blanco.

Figure 5. ***Ficus pseudopalma*** (Earth.com, 2019)

English name: Palm-like fig

Family: Moraceae

Ficus pseudopalma is native to the Philippines but introduced to worldwide tropical and subtropical areas up to elevation 1200 m in secondary forests and the edges of primary forest (Starr, 2003). *F.pseudopalma* is a thin, palm-like, dioecious tree up to 7 m high and 5 cm in diameter. Stem is unbranched with leaf scars. Leaves are alternate, 75 cm long and 15 cm wide with glabrous and shiny on upper surface and pale beneath. Flowers are pollinated by agaonid wasp (Hymenoptera: Chalcoidea: Agaonidae). Figs grow solitary or in pairs with red or purple when ripen (see Figure 5.) (Pancho, 1979).

The tree is cultivated in gardens and parks for its edible young sprouts, fruits and also for ornamental purpose (Tropical Plant Database, 2019). The powdered leaf decoction is used as the treatment for high blood pressure, urinary problems, diabetes, high cholesterol and kidney stones (Santiago *et al.,* 2014)

2.7.6. *Flacourtia indica* (Burm. f.) Merr.

Figure 6. *Flacourtia indica* (National Trust of Australia, 2013)

English name: Governor´s plum

Local name: kakai, bolong, sawa sawa

Family: Flacourtiaceae

F. indica, also known as sawa sawa, is native to Tropical Asia and Madagascar (Martin *et al.*, 1987) and introduced to Central and northern part of South America (Morton, 1987). Tree grows in condition of dry thickets at the lowlands and medium elevations (Seidenschwarz, 1994). It is a shrub or small tree up to 20 meter with 5 cm long spines growing on a trunk and branches. Leaves are oblong or V-shaped with rounded teeth. Flowers are yellowish and only 4 mm wide, growing solitary or in clusters, male and female on separate trees. Fruits are small berries (see Figure 6.), 6-10 mm in diameter, red to purple when ripe, sour and sweet in taste (Krishen, 2013).

Fruit pulp is consumed raw or in jams and jellies. Wood is used in small scale home production (Martin *et al.,* 1987). In Puerto Rico, the tree is used as tall barrier and windbreak. In India, branches are used as fodder for animals. Grounded bark mixed with sesame oil is applied on rheumatic parts and bark infusion is used as gargle. Philippine people use root infusion for treatment of pneumonia and leaf juice is used as remedy for coughs, dysentery and diarrhoea (Morton, 1987).

F. indica is slow-growing species suitable for coppice, resistant to droughts and frosts (Orwa *et al.,* 2009).

2.7.7. *Flacourtia inermis* Roxb.

Figure 7. *Flacourtia inermis* (Flora and Fauna Web, 2013)

English name: Martinique plum

Local name: lovi lovi, botoko

Family: Flacourtiaceae

Flacourtia inermis has unknown origin, but it is cultivated from India, Sri Lanka to Malaysia, Indonesia and Philippines (Alakolanga *et al.*, 2014). The tree grows in hot, humid, tropical lowlands (Martin *et al.*, 1987). It is medium size tree, up to 9 m and 25 cm in diameter. Leaves are oval-shaped 5-20 cm long, spirally arranged on branches. Flowers grow in clusters of 2-8 with greenish petals (National Parks Board, 2013). Fruits are round cherries (see Figure 7.) with light red colour and sour-astringent taste (Alakolanga *et al.*, 2014), containing 4-14 hard, irregular shape seeds about 6 mm wide (Morton, 1987). The tree is cultivated as a fruit tree on sandy and costal soils.

Fruits are eaten fresh or in jellies, pies and jams (Martin *et al.*, 1987). In traditional medicine, roots are used to heal wounds and as a treatment of sore throat. Stomach distensions are traditionally treated with infusion of leaves and roots. Trees are planted in parks and for its ornamental purpose (National Parks Board, 2013).

2.7.8. *Garcinia intermedia* (Pittier) Hammel

Figure 8. *Garcinia intermedia* (Phytoimages, 2011)

English name: Lemon drop mangosteen, Monkey fruit

Local name: waika plum, berba

Family: Clusiaceae

Garcinia intermedia is species native to Central America but introduced along Caribbean islands to Pacific. Natural range of this tree is humid tropical, frost-free,

lowland with annual precipitation to 4 000 mm. This medium sized, evergreen tree grows up to 30 m with dense pyramidal crown. Leaves are dark green, oblong, 6 to 13 cm long with reddish emerge and leathery undersurface. The flowers are white green up to 2 cm in size, blooming in dry season. Fruits are small (see Figure 8.), round, 3 cm long with orange to pale yellow colour and two large seeds (Martin *et al.*, 1987).

Sour fruits are consumed fresh. Lemon drop mangosteen has heavy hardwood which is use for production of fences and tool handles. Tree is widely planted in streets and gardens for its ornamental purpose (Martin *et al.*, 1987).

2.7.9. *Posoqueria latifolia* (Rudge) Roem. & Shult.

English name: Needle-flower tree

Family: Rubiaceae

Figure 9. *Posoqueria latifolia* (Smithsonial Tropical Research Institute, 2010)

Posoqueria latifolia originates in Mexico and Tropical Africa, but it has been introduced to worldwide tropical climate zone (National Park Board, 2013). The small tree is composed of typically short-stalk. Leaves are simple, oblong about 25 cm long, growing opposite on tree branches. The flowers are pollinated by moths, which are only adapted by having proboscises long enough to reach the nectar at the base of the tube (see Figure 9.). The bloom starts by heavy rain following dry period. The fruits are round golden yellow and edible with not very good taste (Barwick, 2004).

In Amazon, people treat clot blood and wounds from poison arrow by using bark of Needle flower tree (National Parks Board, 2013). In the Philippines, fruits are eaten fresh or processed in jellies and jams. Finely grained dense wood is used for production of walking sticks and in some areas. Powdered flowers are used to repel fleas (Barwick, 2004).

2.7.10. *Stelechocarpus burahol* (Blume) Hook. f. &Thomson

Figure 10. *Stelechocarpus burahol* (Flora and Fauna Web, 2013)

Local name: kepel

Family: Annonaceae

Stelechocarpus burahol is native to Indonesia and Malaysia, and introduced to humid evergreen forests of SE Asia. The trunk is stout 25 m tall with rough bark and dense conical crown. Foliage is dark green, emerge reddish, leathery (National Parks Board, 2013). Tree is monoecious with 1 cm long male flowers, growing in groups of 8-16 on the branches. Female flowers, 3 cm long, are held cauliflorously on the trunk. Fruits grow in groups on stem. Fruits are 5-7 cm long, comparable to size of tennis ball, with brown-skin and juicy aromatic flesh, yellowish or pinkish in colour (see Figure 10.). Fruit resembles the fruit of *Manilcara zapota* (Sapotaceae). Seeds are up to 3 cm long, 4-6 pieces per fruit (National Parks Board, 2013). The tree is suitable for higher elevation and resists lower temperatures (Barwick, 2004).

The fruit pulp is a diuretic and is used to prevent and treat kidney inflammation. It is said to cause temporary sterility in women and used by royal ladies as contraceptive (National Parks Board, 2013). Once the fruit is digested, all body excretions, including urine and perspiration, are said to smell strongly of violets (Barwick, 2004).

2.7.11. *Sterculia quadrifida* R. Br.

Figure 11. *Sterculia quadrifida* (Guildford Garden Centre, 2012)

English name: Peanut tree

Local name: Red-fruited kurrajong

Family: Sterculiaceae

S. quadrifida is native to Western Australia and introduced to Philippines. It grows in littoral rainforest close to the sea or rivers at monsoon

forest, in altitude 0-500 m (Cooper and Cooper, 2004). It is evergreen tree, but in cooler areas could be even deciduous (Nicholson, 2007). The height is up to 18 m and diameter to 45 cm (Floyd, 2008). Leaves are alternate, heard-shape to oval 6-27 cm long and 3-18 cm wide. Greenish flowers with lemon scent grow at the end of the branchlets, separate male and female. The fruit calyx is bell-shaped and hairy. Fruits are light red capsules, growing solitary or in groups of five (see Figure 11). Capsules are 5-8 cm long with black seeds about 12-18 mm (Lafrankie, 2010). The tree grows the best in monsoonal forest, vine thickets and seasonal forests (Brown, 1844).

The wood of *S. quadrifida* is used in small range for production of cases and toy. In Australia is wood used by Aborigines as kangaroos nets (Floyd, 2008). In Papua New Guinea, people harvest plants from wild for its edible roots and seeds.

3 Objectives

The main goal of this Master thesis was to evaluate eleven underutilized edible fruit tree species from the Philippines for antioxidant activity by using 2,2-Diphenyl-1-Picrylhydrazyl Radical Assay (DPPH) and Oxygen radical absorbance capacity assay (ORAC). Secondary goal of this thesis was to evaluate these plant samples for phenolic content by using Total phenolic content assay.

Hypothesis:

Many different species rich in body promoting activity have been explored since the discovery of antioxidants. The highest antioxidant activity possessed mostly fruit species from localities in the wild nature and high biodiversity of the species. The Philippine islands have one of the highest species diversities in the world, due to its isolation during the past development periods. Therefore, even those species, which have been chosen for assessment of antioxidant activity in this thesis, were presumed to be active.

4 Materials and methods

4.1 Plant material

Based on previous studies and traditional uses of trees and herbs in Filipino cuisine, 11 fruit tree species have been chosen for the evaluation of their antioxidant activity. The reference specimen sheets with botanical description and natural habitat were elaborated for the purpose of better determination in collection sites. Collection or purchasing of plant samples was held in May 2017.

Figure 12. Plant material collection sites

The plant samples were collected in three localities, namely Los Banos (Laguna province), Sambawan Island (Biliran Islands province) and Manila (Metro Manila province)(marked in Figure 12.), in 5 collection sites (namely in Table 2.) For all species were collected fruits, flowers or leaves with exception of species *F. pseudopalma*, where seeds with pulp and young leaves were collected (see Table 3.). Plant samples were determined by Dr. Pablito Magdalita from University of the Philippines, Los Banos, Prof. Ing. Ladislav Kokoška, Ph.D., and Ing. Johana Rondevaldová, Ph. D. from Department of Crop Sciences and Agroforestry, Faculty of Tropical AgriSciences, Czech University of Life Science, Prague, Czech Republic. For further deposition, voucher specimens (recorded in Table 2.) were elaborated and stored at Czech University of Life Science Prague, except for species *B. luzonica* and, which was purchased on the market and *C. ovatum*, whose nuts could not be processed in

voucher specimen. All plant samples were air-dried and sent by post to Czech Republic for further laboratory research.

4.2 Extract preparation

Dry samples were grounded to mild powder, by using electric mill GM100 (Retsch, Germany) and Tissue Lyser II. (Retsch, Germany). Fine powder was weighted to 1 or 0.5 grams, according to available amount of collected samples (amount of each sample is recorded in Table 2.). Grounded sample was diluted in 30 mL of 100 % methanol and left 24 hours to shake at GFL3005 shaker (Burgwedel, Germany). Subsequently, extract was filtrated and concentrated by Rotary evaporator R-200 (Büchi, Switzerland) at vacuum and 40 ^{o}C, until the sample was dry with lowest possible yield. Dry plant residue was weighted and dissolved in Dimethylsulfoxide (DMSO) to stock solution of concentration 51200 μg/mL and stored at -20^{o}C until use. Extract yield of each plant sample is presented in Table 2.

Table 2. Plant material and extract yield

Scientific name	Family	Voucher specimen number	Local name	Part used	Extract yield (%)	Collection sites
***Argusia argentea* (L.f.) Heine**	Boraginaceae	02511KBFR0	kapal-kapal (tagalog),	leaves	11.1 %	collected on Sambawan Island, Biliran Province
***Broussonetia luzonica* (Blanco) Bureau**	Moraceae	not available	himbabao	inflorescence	12.04 %	bought on market in Manila, Metro Manila Province
***Canarium ovatum* Engl.**	Burseraceae	not available	pili nut	fruits	10.18 %	collected in the garden of the Institute of Plant Breeding, Los Banos, Laguna Province
***Dillenia philippinensis* Rolfe**	Dilleniaceae	02492KBFR8	katmon	fruits	21.54 %	collected in the Mount Makiling forest, Los Banos, Laguna Province
***Flacourtia indica* (Burm.f.) Merr.**	Flacourtiaceae	02494KBFRA	kakai, sawa sawa, bolong, palutan	fruits	57.50 %	collected in the garden of the Institute of Plant Breeding, Los Banos, Laguna Province
***Flacourtia inermis* Roxb.**	Flacourtiaceae	02495KBFRB	lovi-lovi, botoko plum	fruits	38.12 %	collected in the garden of the Institute of Plant Breeding, Los Banos, Laguna Province
***Ficus pseudopalma* Blanco**	Moraceae	02493KBFR9		young leaves	7.78 %	collected in the Dr. R.E. Coronel Fruit Conservation Farm, Los Banos, Laguna Province
***Ficus pseudopalma* Blanco**	Moraceae	02493KBFR9		seeds	7.17%	collected in the Dr. R.E. Coronel Fruit Conservation Farm, Los Banos, Laguna Province
***Garcinia intermedia* (Pittier) Hammel**	Clusiaceae	02496KBFRC	berba, waika plum	pulp	26.06 %	collected in the garden of the Institute of Plant Breeding, Los Banos, Laguna Province
***Posoqueria latifolia* (Rudge) Schult.**	Rubiaceae	02502KBFR0		fruits	20.09 %	collected in the Dr. R.E. Coronel Fruit Conservation Farm, Los Banos, Laguna Province
***Stelechocarpus burahol* (Blume) Hook.f. & Thomson**	Annonaceae	02499KBFRF	kepel	peeled fruit	12.27 %	collected in the Dr. R.E. Coronel Fruit Conservation Farm, Los Banos, Laguna Province
***Sterculia quadrifida* R.Br.**	Sterculiaceae	02519KBFR8	Red-fruited kurrajong	seeds	13.11 %	collected on Sambawan Island, Biliran Islands province

4.3 Chemicals

The plant extracts and subsequent antioxidant activity assays were prepared by using following chemicals and reagents. From Sigma-Aldrich, Prague were purchased: AAPH, Trolox, DPPH, Gallic acid and fluorescein (FL). From Penta (Prague) were purchased: DMSO, F-C and 100 % methanol. From Erba Lachema s.r.o., Brno were purchased: Na_2CO_3 (Sodium carbonate), KH_2PO_4 and from Lach-Ner s.r.o., Neratovice: K_2HPO_4.

4.4 Antioxidant activity evaluation

4.4.1. DPPH - 2,2-Diphenyl-1-Picrylhydrazyl Radical Assay

For assessment of antioxidant activity was used DPPH assay with positive control of Trolox (water-soluble substitution for vitamin E) was based on method of Sharma and Bhat (2009). About 20 μL of stock solution at concentration 51200 μg/mL was diluted in 1980 μL of methanol to create concentration 512 μg/mL. A two-fold serial dilution of each extract was performed via automatized pipetting platform Freedom Evo 100, equipped with a four-channel liquid handling arm (Tecan, Mannedorf, CH) in 96-well microtiter plate. About 75 μL of 100% methanol and then 25 μL of 0.4 mM methanol solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was added into each tested well of microtiter plate to create final volume of 200 μL. Final tested concentrations ranging from 0.125 to 256 μg/mL. Plates were incubated for 30 minutes in dark at room temperature and then measured at 517 nm using Cytation 3 microtitre reader (BioTek,Winooski, VT). Results were calculated by Gen 5 software (BioTek,Winooski, VT) and expressed as IC_{50}.

4.4.2. ORAC - Oxygen radical absorbance capacity assay

The ORAC assay was determined according to Ou *et al.* (2001) with positive trolox control. This method was slightly modified for reasons of better comparison of results with other two assays. Experiment was conducted by using black absorbance 96-well microtitre plate. About 80 μL of plant sample at concentration 102400 μg/mL was

diluted in 1920 μL of buffer (potassium phosphate buffer, 7pH). A two-fold serial dilution of each extract was performed and distilled water boundaries (frame wells around the plate) for better thermal mass stability were prepared in microtitre plate via automatized pipetting platform Freedom Evo 100, equipped with a four-channel liquid handling arm (Tecan, Mannedorf, CH). In each plate was added 150 μL of fluorescein in each well (except boundaries). Microtitre plate was incubated 10 minutes at 37 ^{o}C. The reaction was started with addition of 25 μL of AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride) in each well except boundaries and row 11BCD as control line. The plate was incubated for 90 minutes. The results were read by Cytation 3 microplate reader (BioTek,Winooski, VT) in Gen5 software (BioTek,Winooski, VT) at absorbance wavelengths set to 495 nm. Results were measured as IC_{50} value.

4.4.3. TPC - Total phenolic content assay

TPC assay was determined using modified method of Singleton et al. (1998). About 20 μL of sample (c51200 μg/mL) was diluted in 980 μL of distilled water and then 100 μL of mixture was diluted in 900 μL of distilled water. The 96-well microtiter plate was prepared with 200 μL of Gallic acid diluted with distilled water to 14 different concentrations ranging from 0.015625 to 126 μL/mL, sample dilutions and 25 μL of pure F-C. The plate was inserted in orbital shaker and set to 200 rpm for 10 minutes. Reaction was started with addition of 75 μL of Na_2CO_3 (12 %). Microtiter plate was kept in dark at 37 ^{o}C for 2 hours. The microtitre plate was measured by Cytation 3 microplate reader (BioTek,Winooski, VT), wavelength 700 nm and presented in Gen5 software (BioTek,Winooski, VT). The results expressed as Gallic acid equivalent (GAE mg/g).

4.5 Data processing

The plant samples were tested *in vitro*, using two methods for assessment of antioxidant activity DPPH, ORAC and one method for assessment of Total phenolic content, using method TPC by Folin-Ciocalteu. The plant samples were tested in triplicate for each method and in three independent experiments. Results were expressed as mean value with standard deviation (mean ± SD).

The data of DPPH and ORAC assay were expressed as IC_{50}, *in vitro*. IC_{50} value represents concentration of drug/extract needed for at least 50 % inhibition. The data of GAE Values of IC_{50} and GAE were performed in software Gen 5 of Cytation 3 microplate reader (BioTek,Winooski, VT). Further data were processed in Microsoft Office Excel 2010. Statistical analyses of linear correlation between DPPH and TPC, ORAC and TPC were analysed by using Pearson Correlation Coefficient in Microsoft Office Excel 2016.

5 Results

5.1 DPPH

The strongest antioxidant activity was determined for *Stelechocarpus burahol* with IC_{50} value of 90.99±12.63 μg/mL. *Broussonetia luzonica* possessed weaker antioxidant activity with IC_{50} 253.65±13.07 μg/mL. No other plant extract exhibited antioxidant activity even at the highest concentration tested. Table 3. presents values of antioxidant activity for DPPH assay.

Table 3. Results of DPPH assay

Scientific name	IC_{50}(μg/mL)
***Argusia argentea* (L.f.) Heine**	>268,8
***Broussonetia luzonica* (Blanco) Bureau**	253.65±13.07
***Canarium ovatum* Engl.**	>268.8
***Dillenia philippinensis* Rolfe**	>268.8
***Ficus pseudopalma* Blanco – leaves**	>268.8
***Ficus pseudopalma* Blanco - seeds with pulp**	>268.8
***Flacourtia indica* (Burm.f.) Merr.**	>268.8
***Flacourtia inermis* Roxb.**	>268.8
***Garcinia intermedia* (Pittier) Hammel**	>268.8
***Posoqueria latifolia* (Rudge) Schult.**	>268.8
***Stelechocarpus burahol (*Blume) Hook.f. & Thomson**	90.99±12.63
***Sterculia quadrifida* R.Br.**	>268.8
Trolox	9.19±2.54

IC_{50} – the half maximal inhibitory concentration (μg/mL)

5.2 ORAC

Strongest antioxidant activity was determined by ORAC assay was possessed at *B. luzonica* with IC_{50} value of 32.2±12.9 μg/mL and *S. burahol* with IC_{50} value 37.9±8.7 μg/mL. Seeds with pulp of *F. pseudopalma* exhibit values of IC_{50} 106.7±25.2 μg/mL, whereas young leaves of *F. pseudopalma* possessed weaker activity of IC_{50} 132.2±8.1 μg/mL. Species *S. quadrifida* exhibited 152.3±9.4 μg/mL, *P. latifolia* 166.68±21.9 μg/mL, *F.indica* 203.7±25.4 μg/mL, whereas *F. inermis*, as a species with the lowest determined antioxidant activity, possessed only 236.6±14.6 μg/mL. Species *A. argentea, C. ovatum, D. philippinensis* and *G. intermedia* did not exhibit any

antioxidant activity even at the highest concentrations. Table 4. presents evaluation of all species by ORAC assay with IC_{50} values and standard deviations.

Table 4. Results of ORAC assay

Scientific name	IC_{50}(μg/mL)
***Argusia argentea* (L.f.) Heine**	>268.750
***Broussonetia luzonica* (Blanco) Bureau**	32.2±12.9
***Canarium ovatum* Engl.**	>268.750
***Dillenia philippinensis* Rolfe**	>268.750
***Ficus pseudopalma* Blanco – leaves**	132.2±8.1
***Ficus pseudopalma* Blanco - seeds with pulp**	106.7±25.2
***Flacourtia indica* (Burm.f.) Merr.**	203.7±25.4
***Flacourtia inermis* Roxb.**	236.6±14.6
***Garcinia intermedia* (Pittier) Hammel**	>268.750
***Posoqueria latifolia* (Rudge) Schult.**	166.68±21.9
***Stelechocarpus burahol* (Blume) Hook.f. & Thomson**	37.9±8.7
***Sterculia quadrifida* R.Br.**	152.3±9.4
Trolox	7.9±1.2

IC_{50} – the half maximal inhibitory concentration (μg/mL)

5.3 TPC

The highest phenolic content was determined at *B. luzonica*, where value of GAE per gram of extract was 133.0 mg/g, with standard deviation 5.6. Sample with lower phenolic content, but also significant, was determined at *S. burahol* with value of Gallic acid equivalent of 72.2±4.5 mg/g. No other plants extract exhibit higher TPC. Table 5. shows results of all evaluated plant extracts.

Table 5. Results of TPC assay

Scientific name	GAE(mg/g)
***Argusia argentea* (L.f.) Heine**	16.7±5.1
***Broussonetia luzonica* (Blanco) Bureau**	133.0±5.6
***Canarium ovatum* Engl.**	23.1±4.5
***Dillenia philippinensis* Rolfe**	18.1±2.3
***Ficus pseudopalma* Blanco – leaves**	15.8±0.9
***Ficus pseudopalma* Blanco - seeds with pulp**	14.2±4.1
***Flacourtia indica* (Burm.f.) Merr.**	22.3±5.7
***Flacourtia inermis* Roxb.**	25.2±1.6
***Garcinia intermedia* (Pittier) Hammel**	15.6±1.9

***Posoqueria latifolia* (Rudge) Schult.**	18.9±3.3
***Stelechocarpus burahol* (Blume) Hook.f. & Thomson**	72.2±4.5
***Sterculia quadrifida* R.Br.**	26.6±3.2

GAE – gallic acid equivalent (mg/g)

Linear correlation coefficients between DPPH values and TPC values indicates weak correlation ($r<0.377$). The results of DPPH assay are significant at $P>0.05$. Linear correlation coefficients between ORAC and TPC was evaluated as strong ($r<0.933$) and significant at $P<0.01$.

6 Discussion

The antioxidant evaluation of selected fruit tree species traditionally eaten at the Philippines revealed significant values at two species: *S. burahol* and *B. luzonica*. Other evaluated species possessed just weak or no activity. Also estimation of TPC exhibited high value at *S. burahol* and *B. luzonica*. According to the studies of Tisnadjaja *et al.* (2006) *S. burahol* fruit was previously tested for antioxidant activity with DPPH assay. The method showed low IC_{50} at n-butanol and ethyl acelate extract. Subsequently, flower part of *S. burahol* was tested with same method in ethyl acelate extract and possessed lower antioxidant activity, than ethyl acelate extract of the fruit. In comparison of results for fruit of *S. burahol* in n-butanol and ethyl acelate with methanol extract, used in this thesis, methanol extract reports the highest antioxidant activity. In other study of Nurmalitasari *et al*. (2014), tested parts of *S. burahol* were leaves. The leaves were tested with DPPH assay and reached values of IC_{50} 190.0 μg/mL. *In vitro* studies of kepel leaves possessed antioxidant activity related to higher flavonoid content. In comparison of both, fruit, tested in this thesis and leaf plant extract, kepel fruits exhibited higher antioxidant activity.

The inflorescence plant extract of *B. luzonica,* as extracts with second highest values measured, was not recorded in scientific journals and articles as species tested to antioxidant activity. Many studies prove the anticancer, antimicrobial and antioxidant activity of other species of the genus *Broussonetia (*Wang *et al.* 2010; Park *et al., 2013*), often used in Chinese medicine (Casuga *et al.*, 2016). In study of Sun *et al.* (2012), fruits of *B. papyrifera* were tested for TPC and antioxidant activity with DPPH assay. The method of DPPH was determined in two extracts, aqueous and ethanol. The results showed higher antioxidant activity at aqueous extract and the antioxidant activity of extracts was positively associated to their total phenolic content. This study opens the possibility of further testing of *B. luzonica* in aqueous extract solution, which could result in higher antioxidant activity.

According to our best knowledge, fruits of *S. quadrifida* were not found in scientific articles to be tested for antioxidant activity. Lulan *et al.* (2018) tested the roots of *S. quadrifida* with DPPH assay in methanol extract and found it to be active with values of IC_{50} 3.11 μg/mL. Also determination of phenolic content was made with values of GAE

6.19 mg/g. Otherwise, evaluation of fruit extract of *S. quadrifida* determined in this thesis did not possess any antioxidant activity with DPPH assay and only weak activity with ORAC assay, content of phenolic compound was low.

Also the leaf extract of *F. pseudopalma,* evaluated in this thesis, did not exhibit any antioxidant activity in DPPH assay and only weak activity in ORAC assay (132.2 μg/mL). Joshua and Librado (2015) were evaluating antioxidant activity of leaves by DPPH assay and estimation of TPC, both in 5 different extracts fractionated following a modified Kupchan´s partition scheme, using solvents of increasing polarity: petroleum < ether < chloroform < ethyl acetate < n-butanol < water. Surprisingly, the strongest antioxidant activity and the highest phenolic content were determined in ethyl acelate extract with IC_{50} 0.71 μg/mL and at GAE 2.93mg/g.

The testing of antioxidant activity with DPPH and ORAC assay, tested in this thesis, did not determine any activity at species *D. philippinensis* and only low content of phenolic compounds. Barcelo (2015) have tested this species for antioxidant activity with DPPH assay and TPC. The results showed high antioxidant activity (91.13 %) and low content of phenolic compounds. Barcelo explains the high antioxidant activity of *D. philippinensis* by presence of other secondary metabolites: alkaloids, steroids, saponins and tannins. The difference of antioxidant activity between the results in study of Barcelo and results in this thesis can be caused by different extract preparation: dried samples for this thesis and fresh samples in the study of Barcelo.

Tisnadjaja *et al.* (2006) evaluates *S. burahol* as an endangered Indonesian species. The economic value of this species is low and people neglect to plant it. The main reasons of Mr. Tisnadjaja and collegues for assessment of antioxidant activity at this species, was to find additional value for replanting the tree and saving the species in the country of the origin. Analogous situation is happening in the Phillipines, where the indigenous species, used for ages in home cuisine and home medicine, are replaced by fruit and timber trees with high commercial value. *B. luzonica* and *F. pseudopalma* have been told to be endemitic species of the Philippine lowlands. Even for preservation of this species in its origin, *B. luzonica* could be suitable for homegardens and lowland multi-storey agroforestry systems. *S. burahol* as a species introduced to the Philippines

from Malaysian and Indonesian rainforest is suitable for forest sites and its replantation (Barwick, 2004). This tree species is perfect as shading tree in Taungya agroforestry systems, for production of fruits and even after the closing of canopy it could perfectly adapt to forest succession.

The evaluation of antioxidant activity in this thesis revealed strong antioxidant activity only at two species (*S. burahol, B. luzonica*) and weaker antioxidant activity with ORAC assay at three others plant extracts (*F. pseudopalma* - leaves, *F. pseudopalma* – seeds, *Sterculia quadrifida*). The values of antioxidant activity and total phenolic content of selected species is compared to species with proven strong antioxidant activity and species above mentioned, found in scientific articles are marked in Table 6.

Therefore, the hypothesis of this thesis was proved just partially. Also subsequent TPC method possessed high content just at two species (*S. burahol, B. luzonica*). The antioxidant activity of the species is not always related to its phenolic content. Many other compounds, such as tannins, steroids and alkaloids could be significant for high antioxidant activity. Also different extract solution, conditions for growth, climate and soil conditions changes the results of complex assessment antioxidant activity of species.

Table 6. Antioxidant activity and total phenolic content of tested species in compare to species found in scientific articles

Scientific name	Local name	Extract solution	Part used	DPPH IC_{50}(µg/mL)[1]	ORAC IC_{50}(µg/mL)	TPC GAE(mg/g)[2]	Reference
***Argusia argentea* (L.f.) Heine**	kapal-kapal (tagalog),	methanol	leaves	>268,8	>268.750	16.7±5.1	
***Broussonetia luzonica* (Blanco) Bureau**	himbabao	methanol	inflorescence	253.7±13.1	32.2±12.9	133.0±5.6	
***Canarium ovatum* Engl.**	pili nut	methanol	fruits	>268.8	>268.750	23.1±4.5	
***Dillenia philippinensis* Rolfe**	katmon	methanol	fruits	>268.8	>268.750	18.1±2.3	
***Ficus pseudopalma* Blanco**	kakai, sawa sawa,	methanol	young leaves	>268.8	132.2±8.1	15.8±0.9	
	bolong, palutan	ethyl acelate	young leaves	0.71	-	2.9	Joshua and Librado (2015)
***Ficus pseudopalma* Blanco -**	lovi-lovi, botoko plum	methanol	seeds	>268.8	106.7±25.2	14.2±4.1	
***Flacourtia indica* (Burm.f.) Merr.**		methanol	fruits	>268.8	203.7±25.4	22.3±5.7	
***Flacourtia inermis* Roxb.**		methanol	fruits	>268.8	236.6±14.6	25.2±1.6	
***Garcinia intermedia* (Pittier) Hammel**	berba, waika plum	methanol	pulp	>268.8	>268.750	15.6±1.9	
***Posoqueria latifolia* (Rudge) Schult.**		methanol	fruits	>268.8	166.68±21.9	18.9±3.3	
***Stelechocarpus burahol* (Blume) Hook.f. & Thomson**	kepel	methanol	peeled fruit	91.0±12.6	37.9±8.7	72.2±4.5	
		methanol	leaves	190.0			
***Sterculia quadrifida* R.Br.**	Red-fruited kurrajong	methanol	seeds	>268.8	152.3±9.4	26.6±3.2	
		methanol	roots	3.11	-	6.19±1.22	Lulan *et al.* (2018)
Trolox			-	9.2±2.5	7.9±1.2	-	
Phyllanthus emblica	Amla, Nelli	aquaeous	fruits	0.02±0.00	-	295.9±3.6	Jayathilake *et al.* (2016)

[1] IC50 - IC50 – the half maximal inhibitory concentration (µg/m)
[2] GAE – gallic acid equivalent (mg/g)

7 Conclusion

In this Master thesis, antioxidant activity of eleven edible tree species from the Philippines have been determined by two methods DPPH, ORAC and TPC was estimated. The results showed that the highest antioxidant activity within all selected species possessed *S. burahol* with IC_{50} 90.99 μg/mL (DPPH assay) and IC_{50} 37.9 μg/mL (ORAC assay). Second highest antioxidant activity exhibited *B. luzonica* with values of IC_{50} 253.65 μg/mL (DPPH assay) and IC_{50} 32.2 μg/mL (ORAC assay). Even at TPC assay, the highest values possessed *B. luzonica* with GAE 133.0 mg/g and *S. burahol* with GAE 75.2 mg/g. No other plant extract possessed higher antioxidant activity, within all assays. Statistical analyses proved that antioxidant activity of evaluated plant species was mainly caused by higher content of phenolic compounds, but there are also other compounds influencing antioxidant activity of extracts.

Many different studies tested antioxidant activity of various plant parts of *S. burahol*, but according to our best knowledge, fruit parts were never tested with ORAC assay. In comparison of plant parts tested and extract solution used, the highest activity was determined in methanol extract of fruit (tested in this thesis). The inflorescence of *B. luzonica* was never tested for antioxidant activity, but the fruits of genus *Broussonetia* were found in scientific article to be active. For further antioxidant activity evaluation is recommended to test fruit part.

The result is a valuable reference of antioxidant properties from Philippine plants used in traditional cuisine, which serves as scientific value of antioxidant source in the Philippines. The tree species with higher antioxidant activity determined, could be more introduced to agricultural and forest composition and can be used in agroforestry systems such as lowland multi-storey systems (*B. luzonica*), Taungya system (*S. burahol*) and also in home gardens.

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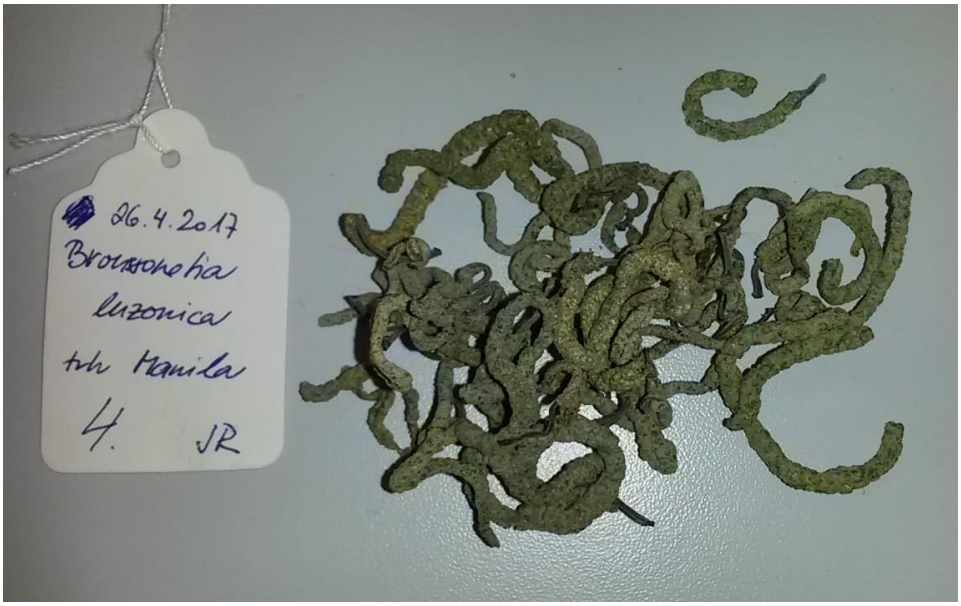
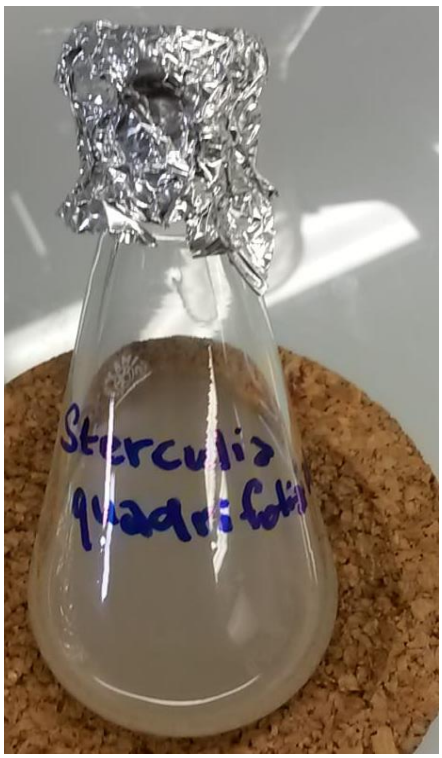
10 The appendices

Annex I. Dry sample of the species *Canarium ovatum*

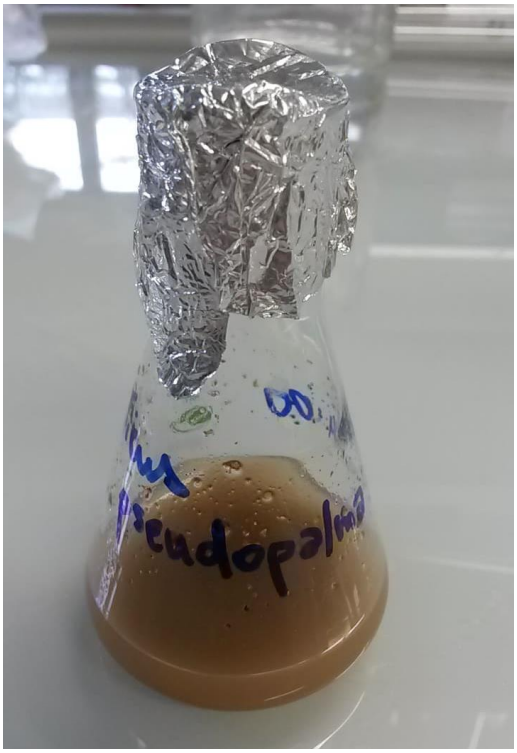
Annex II. Dry sample of the species *Broussonetia luzonica*



Annex IV. Methanol sample after filtration



Annex III. Methanol dillution after shaking



Annex V. Assessment of TPC