ISOLATION AND DETERMINATION OF ANTI-NUTRITIONAL COMPOUNDS FROM ROOT AND SHELLS OF PEANUT (*Arachis Hypogaea*)

By

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ABSTRACT

ISOLATION & DETERMINATION OF ANTI-NUTRITIONAL COMPOUNDS FROM ROOT AND SHELLS OF PEANUT (*Arachis Hypogaea*)

SIM EE WEI

Tremendous amount of agricultural waste to environment and high cost of conventional feedstuffs has led to the evaluation of peanut shells and root for suitability of peanut by-products as animal feedstuffs considering anti-nutritional compounds present in these by-products. Anti-nutritional factors include phytic acid, saponins and alkaloids were determined in Shandong peanut shell, Menglembu peanut shell and peanut root. Phytic acid, total steroidal saponins and total saponins were determined by spectrophotometric assay. Total alkaloids content was quantified by gravimetric analysis; Qualitative evaluation of alkaloids was conducted through thin layer chromatography. The phytic acid level of peanut shell and roots ranged from 0.11 – 0.17 g/ 100g. The concentration of total steroidal saponins and total saponins content in peanut shells and root are 0.90 – 2.57 mg/ 10g and 12.3 – 21.2 mg/ 10g respectively. Only Berberine was identified in peanut root and total alkaloids content of peanut shells and root are within the range of 4 – 24%. In brief, the level of phytic acid and saponins in peanut shells and root unlikely exerts anti-nutritional effects and toxicity to living organisms if used as animal feedstuffs. The toxicity threshold of alkaloids is yet to be determined in future studies.

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Last but not least, my sincere thanks to my beloved family for their mental support, understanding and encouragement given by them all over the project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree in UTAR or other institutions.

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SIM EE WEI

APPROVAL SHEET

This project report entitled "I**SOLATION AND DETERMINATION OF ANTI-NUTRITIONAL COMPOUNDS FROM ROOT AND SHELLS OF Arachis Hypogaea"** was prepared by SIM EE WEI and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biochemistry at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **SIM EE WEI** (ID: **08 ADB 06141**) has completed this final year project entitled **"ISOLATION AND DETERMINATION OF ANTI-NUTRITIONAL COMPOUNDS FROM ROOT AND SHELLS OF PEANUT (*Arachis Hypogaea*)"** supervised by **Dr. CHANG YING PING** from the Department of Chemical Science, Faculty of Science.

I hereby to give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

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LIST OF ABBREVIATION

USDA	United State Department of Agriculture
UVB	Ultraviolet B
UVC	Ultraviolet C
DDT	Dichlorodiphenyltrichloroethane
TCA	Trichloroacetic acid
HCl	Hydrochloric acid
HNO_3	Nitric acid
HIV	Human Immunodeficiency Virus
Nm	Nanometer
HPLC	High-Performance Liquid Chromatography
TLC	Thin Layer Chromatography
GLC-MS	Gas-Liquid Chromatography – Mass Spectrometry
GC	Gas Chromatography
NMR	Nuclear Magnetic Resonance
ELISA	Enzyme-Linked Immunosorbent Assay
RIA	Radio Immunodiffusion Assay
λ_{max}	Lambda Maximum
R_f	Retention Factor
℃	Degree Celsius
g	Gram
w / w	Weight per weight
mL	Millilitre
M	Molar

$Fe(NO_3)_3.9H_2O$	Iron (III) nitrate nonahydrate
KSCN	Potassium thiocyanate
Phytate - P	Phytate – phosphate
mins	Minutes
N	Normality
µg	Microgram
mg	Milligram
µL	Micro litre
rpm	Rotation per minute
$FeCl_3$	Iron (III) chloride
R^2	Regression coefficient
AG	Analytical Grade
v/v/v	Volume per Volume per Volume
Apo B	Apolipoprotein B
ESI/ MS-MS	Electrospray Ionization – tandem Mass Spectrometry
Eds.	Editors
et al.	et alia (and others)

CHAPTER 1

INTRODUCTION

Agricultural waste is any materials left over from agricultural process, farming and livestock operations include animal manures, oilseed, grain, vegetable and fruit harvest residues. Stalk, bran, root and shell are the common example of agricultural by-product for oilseed crop residues. Legume family is one of the largest families of plant kingdom that contributes agricultural wastes in form of oilseed harvest residues. Peanut is the second most important legume in the world on the basis of total production after soybean (Redden, Chen & Sharma, 2005).

High production of agricultural waste accompanied with high production of agricultural products. For instance, China as the biggest agricultural country in the world produces over 800 million tons of agricultural waste annually (Yue, Zhang, Zhao & Luo, 2009). USDA reported that total world production of peanut increase from 31.48 to 33.73 million metric tons of shell peanuts with increment of 7.13% in year 2010/2011 (Georgalos, 2010).

Over production of agricultural waste becomes a serious issue to environment and nation. The common methods in handling oilseed harvest residues such as peanut by-products are incineration, incorporation in soil and land dumping. However, incineration will emit smokes or particulates which cause air pollutions; incorporation of agricultural waste in soil will influence physical, biological and chemical properties of soil (Osterli, 1972). These methods are

not the good solutions in managing tremendous amount of peanut by-products. Thus, this poses a critical problem in waste management system and subsequently causes environmental pollution if the agricultural waste is not managed properly (Westendorf, 2000).

Effective ways of managing agricultural waste are crucial in protecting the environment, maintaining water quality and ensuring food quality. Transformation of agricultural waste to value-added raw material, ingredient or product is one of the best alternatives in curbing agricultural waste problems and reducing waste management costs (Ebine, 1973; Crickenberger & Carawan, 1996; El-Haggar, 2001; Lesdema, Santos & Briagas, 2004; Kyriazakis & Whittenmore, 2006; Dongmeza *et al.*, 2009; Maheswari *et al.*, 2010). National Research Council of United State (1989) described underutilized materials as agricultural wastes which have extensive potential value as animal feedstuffs.

Utilization of peanut by-products as animal feedstuffs offers several benefits. First and foremost, it is cost-saving compared to conventional feedstuffs (Lesdema *et al.*, 2004). Conventional feedstuffs such as fish, crustacean and animal meal are relatively expensive than plant-based feedstuffs (El Sayed, 2004). Sharp rises of animal feed market prices since 1973 has encouraged livestock producers to seek cost-saving alternatives, which is by-products from oilseed or legumes (Ebine, 1973). National Research Council (U.S) Subcommittee on Underutilized Resources as Animal Feedstuffs reported the availability of agricultural waste enable it becomes potentially valuable feedstuffs. Total feed use in 2010/11 is forecast to be increased by 1.1 million

tons to 11.5 million tons. Agricultural waste available in large quantity accompanied with production of agricultural products such as peanut per annum. Thus, this provides an adequate supply of agricultural by-products for underutilized animal feed formulation.

Nutrition profile that offers by oilseed harvest residues offers another advantage. Agricultural by-products consist of satisfactory level of energy, minerals, proteins and fibers or even antioxidant that are essential for animal (Crickenberger & Caravan, 1996). Available data indicates that nutrition profile of animal feedstuffs influences animal performance. Besides, the deficiency of animal feedstuffs as faced by Egypt can be solved by application of agricultural waste as animal feeds (El-Haggar, 2001).

However, the presence of anti-nutritional factors such as phytic acid, lectins, saponins, alkaloids, gluconosilates, phyto-estrogens and other anti-vitamins in plant-based feeds limits its usage in animal feedstuffs as well as aquaculture feeds (Maheswari *et al.*, 2010). Nutritionists believe that anti-nutritional factors responsible to influence physiological characteristic and retard growth of animals (Lesdema *et al.*, 2004). A study has confirmed the harmful effects of anti-nutrients to fish by causing growth retardation (Johnson, Gee, Price & Curl & Fenwick, 1986, Bureau, Harris & Cho, 1998, Shimoyamada, Ikedo, Ooutsubo & Watanabe, 1998, Dongmeza, Steinbronn, Francis, Frocken & Becker, 2006). Anti-nutritional factors are naturally-occurring compounds that exert anti-nutritional effect, anti-physiological effect and limit the nutrients availability to living organism (Felix & Mello, 2000; Panhwar, 2005). This

study investigates the presence of anti-nutritional factors in peanut by-products and evaluates its suitability as animal feedstuffs.

Peanut by-products that involved in this study are peanut shell and peanut root. The root system (Figure 1.1) of a peanut plant comprised of a main or “tap” root and secondary roots. Each peanut plant consists of a main or “tap” root whereby the smaller, secondary root developed from it. Peanut roots can be serves as cuisine, biofuel, lotion, conditioner, shampoo and body wash. Peanut root has been found as a potential source of antioxidant resveratrol. Root system of peanut plant conducts nitrogen fixation and absorbs water from soil to keep plant refreshing (Chen, Wu, Robin & Chiou, 2002).

There are two varieties of peanut shell being analyzed, which are Shandong peanut (Figure 1.2) and Menglembu peanut (Figure 1.3). Shandong Peanut often known as “Shandong Big Peanuts” or even “Da Li (Darlie)”. Shandong peanut can be characterized as plump grain size, large size, excellent taste and high nutrition. Shandong peanut evolved from two species which can be differentiated by shape. These two kernels having two different shapes: one is round Spanish type, which known as HSIAOBAISHA (Xiao Bai Sha); the other one is oblong Virginian type and called TSUJI. Shandong peanut was named as this type of peanut was first originated and cultivated in eastern China’s Shandong province. Shandong province is suitable for Shandong groundnuts cultivation due to its mild climate and good quality of soil in mountainous area which enriches with phosphate as well as free of aflatoxin contamination.

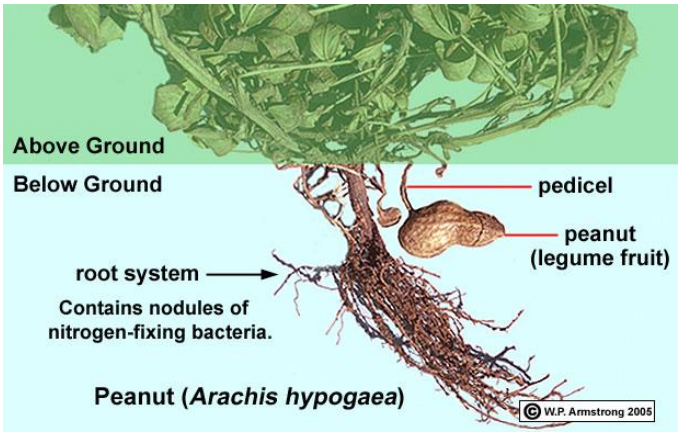


Figure 1.1: Root system of peanut plant.

Figure 1.2: Shandong peanut.

Figure 1.3: Menglembu peanut

Menglembu peanut is another cultivar that being discovered and developed in Menglembu town, Perak, Malaysia. Menglembu symbolises the procedure of groundnuts processing rather than the name of town. It is famed for its crunchy texture, tasty flavour, and aromatic smell. Besides, compare with other peanut cultivars, Menglembu groundnut contains less oil. In comparison to Shandong groundnut, the granule size of Menglembu peanut is smaller and its colour of shell is lighter.

The anti-nutritional factors being analysed in this study are phytic acid, saponins and alkaloids. Phytic acid is a common constituent in plant materials that acts as a chelator of mineral ions and interferes with mineral bioavailability in digestion system of living organism (Morris, 1986). Saponins present in different plants and reported to be toxic or growth retarding for fish (De la Higuera, Garcia-Gallego, Sanz, Cardenete, Suarez & Moyano, 1988). Saponins also affect the permeability of the small intestinal mucosal cells and subsequently influence active nutrient transport. Saponins exerts anti-physiological effects by inhibit digestive enzymes and protein degradation process. Alkaloids are known to toxic compounds to living organism and impart anti-physiological effects on neurological activities. Over consumption of alkaloids will cause paralysis as well as rapid heartbeat, and leading to death in fatal case (Makkar, Becker & Siddhuraju, 2007).

This study was designed to investigate the presence of anti-nutritional factors in peanut by-products through qualitative and quantitative evaluation. The objective of this project is to determine the suitability of peanut by-product as

animal feedstuffs by comparing the level of anti-nutrients with theoretical value that induces toxicity in living organisms.

The rationale for undertaking this study illustrated as below:

i. Peanut by-products are potential animal feedstuffs due to its cost-saving, satisfactory level of nutritional values and bioavailability.
ii. Living organism could consume peanut by-products if they consist of low level of anti-nutritional factors without significant adverse effect to their growth and physiological characteristic.

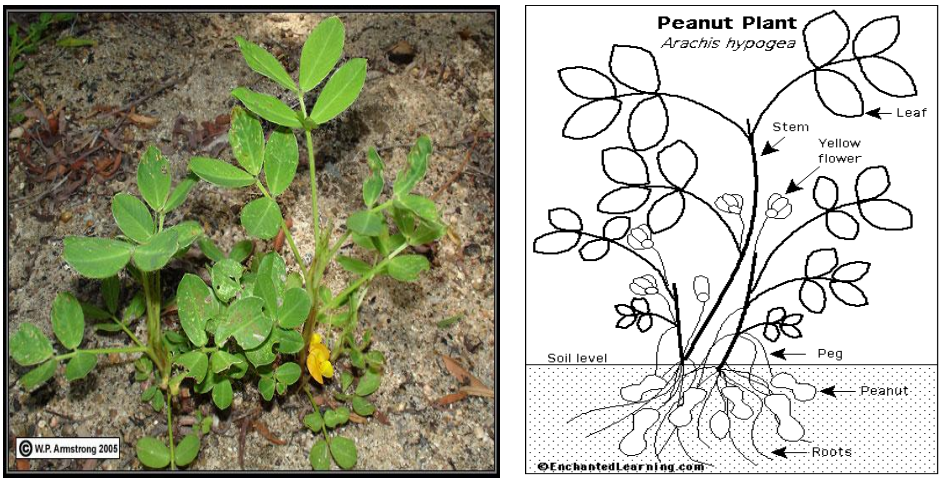
CHAPTER 2

LITERATURE REVIEW

2.1 Peanut Plant

A peanut plant (Figure 2.1) is a unique plant where its flower grows above the ground, while its pod develops to underground (Lamb & Johnson, 2011). The peanut plant composed by leaf, stem, peg, flower, seed and root. The scientific name of peanut is Arachis *Hypogaea*, in which "*Hypogaea*" refers to "under the earth". The other names of peanut includes groundnut, monkey nut, earthnut, gobber, gobber pea, peanut vine, potato bean, wild bean, earth-ball, truffle, Bambara, Chang Sheng Guo (Long-life nuts), pygmy nut and pig nut.

Peanut is the second most important legume in the world on the basis of total production after soybean (Redden *et al.*, 2005). The Peanut Institute publicized that peanuts and peanut products gain the highest popularity in market of United States and comprise 67% of all nut composition (Figure 2.2). George Washington Carver (1865 – 1943) discovered 300 derivatives products from peanuts, including plastics, soap, cosmetic, nitroglycerin, dyes, paints and flour. Unlike peanut seed as important constituent for human consumption and food manufacturing, peanut root and shell are common by-products that being eliminated from food processing.



(a) (b)

Figure 2.1: (a) A peanut plant with a yellow flower grows at the base; (b) A peanut plant with its labelling organs.

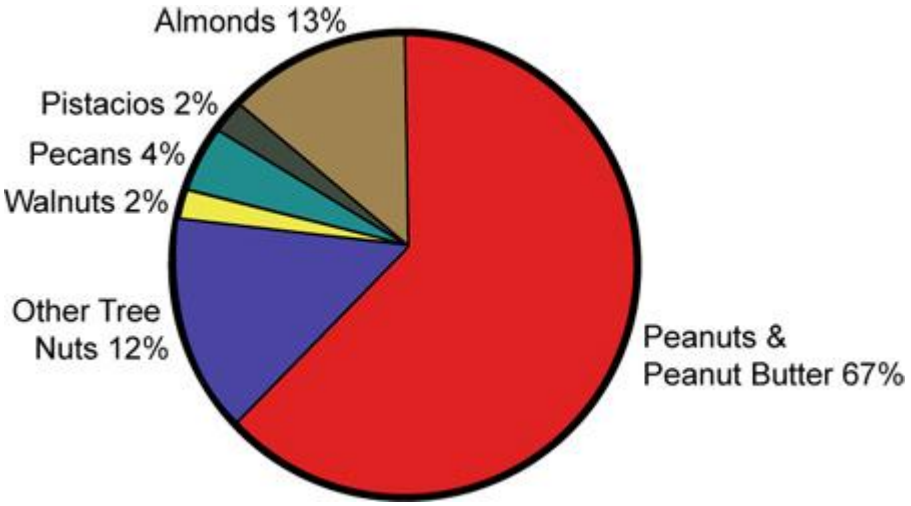


Figure 2.2: Popularity of peanut and its products in market of United States.

2.1.1 Peanut root

Peanut root system (Figure 2.3) comprised of a main or "tap" root, in which the smaller, secondary roots developed from it. The main root grows close to 18 inches into the soil, whereas secondary roots grow 4 to 6 inches into the ground (Biles, 2010). In early embryonic development of peanut kernel, root cap

(Figure 2.4) grows on the tip of new root to protect the meristematic root tip as it pushes into the ground (Armstrong, 2009). Root system of peanut plant conducts nitrogen fixation, in which fixing the nitrogen from the air and feed the plant. The capability of nitrogen-fixing bacteria to conduct nitrogen fixation makes peanut plant as an ideal plant for the rotation crop. Thus, peanut root system can enrich the soil and maximise the production of crop.

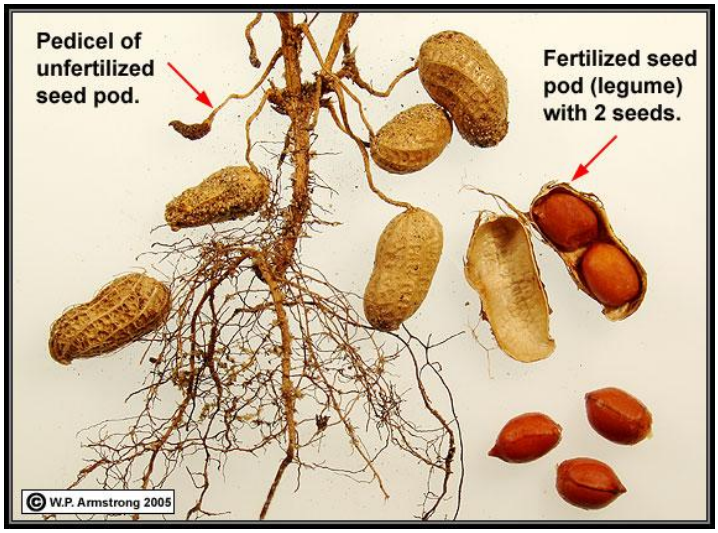
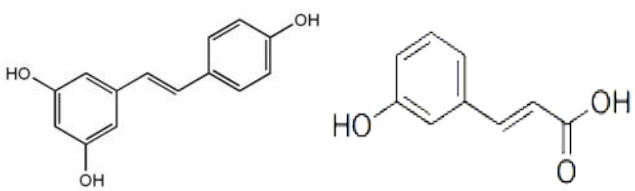


Figure 2.3: A peanut plant with seed-bearing dry fruits or pods.

Figure 2.4: The germinated peanut seeds showing the root cap.

Peanut roots can be used widely in different fields. In ready Chinese cuisine, peanut roots always served as vegetable or soup. Some people believed that peanut root soup can enhance one's immunity, promote better health, decreases allergy problem in youth or increase height of children. However, all of these beliefs still required further investigation as there is no any scientific research that proves this statement (Chia, 2006).

Besides, peanut root has been reported to be a good source of antioxidant resveratrol (Chen *et al.*, 2002). Resveratrol or 3,4',5-trihydroxystilbene (Figure 2.5) classified as phytochemical and is a naturally occurring plant defence compound which functions to protect plant from diseases. Peanut roots contain caffeic acid that stimulate hair roots and sustains skin elasticity (Garland, 2004; Keoke & Porterfield, 2005). Caffeic acid or hydroxycinnamic acid (Figure 2.5) is a naturally occurring organic compound that possesses anti-cancer property and anti-inflammatory properties that protect skins cells during exposure of ultraviolet radiation, specifically UVB and UVC radiation. Thus, peanut roots may serve as a lotion, body washes, conditioners and shampoos in body care industry even as a cosmetic product.



(a) (b)

Figure 2.5: Chemical structure of (a) antioxidant resveratrol; (b) hydroxycinnamic acid

2.1.2 Peanut shell

Peanut shells also known as peanut hulls, similar with peanut roots, are classified as low value agricultural wastes or agricultural by-products. However, new technology and innovation has converted peanut hulls to a wide range of applications. Yue *et al.* (2008) stated that peanut shells which are agricultural by-products are the most available biomass resources in China. This unique renewable energy resource has a high potential to be an alternative for fossil fuels. Other applications of peanut shell include adsorbent for heavy metals, development of fertilizer carriers, plastic composite materials, insulation block, bedding purpose, floor-sweeping compounds, manufacture of linoleum and dynamite, preparation of magnesium tiles and plaster (Clay, 1941).

In early 1940', literature sources have reported the utilization of peanut shell as animal feedstuffs, such as dogs, cattle and horses (National Research Council, 1989) as peanut shells or peanut hulls was found to be contained moderate level of protein (8%) as well as high level of crude fiber and lignin (Clay, 1941; Utley & McCormick, 1972). For instance, some of south western firms in United State prepare cattle feed by mixing molasses and peanut hulls in the proportion of 80:20 with a little of salt added into it. This molasses feed for cattle contains 6 – 7% of protein. As for feedstuffs for horses, peanut hulls contain higher amount of crude protein as compared with cottonseed hulls.

Jankowski (2010) reported that the high levels of cellulose and crude fiber of peanut shells can be incorporated into food supplement products to boost up

dietary fiber intake of consumer. This subsequently reduces several disorders such as colon cancer, constipation, diabetes, ischemic heart disease, diverticular disease and other gastrointestinal disorders. Peanut hulls occasionally serve as fiber source for dog. Due to this, peanut hulls also known as cheese rind "cheese", "vegetable fiber" and feathers "processed poultry protein" in pet food industry (Wsong, 1992). However, this might cause toxicity to dogs as peanut hulls are susceptible to fungus and aflatoxin. Aflatoxin is a kind of toxin or poison that produced by a strain of fungus, *Aspergillus flavus*. The George Mateljian Foundation (2011) revealed that aflatoxin is twenty times carcinogenic than DDT that will lead to lowered intelligence and mental retardation.

Lindsey & Turner (1975) found out that there are various anti-nutritional factors present in peanut shells, cotyledons and skin such as phenols, tannins and related pigment. The presence of condensed tannins gives brown coloration of peanut hulls. Whereas, Daigle, Conkerton, Hammons & Branch (1988) revealed that there is a difference in the nature of flavonoids in immature and mature shell. In contrast to fruit or peanut seeds, most of flavonoids as well as tannins concentrated in peanut hulls and seed coats. In mature peanuts, the percentage of condensed tannins in different parts of peanut: seed coats (6.04%), hulls (0.43%) and fruit (0.04%).

2.2 Anti-Nutritional Factors

2.2.1 Classification of anti-nutritional factors

Anti-nutritional factors are naturally occurring compounds that classified under a broad group of secondary metabolites. Felix and Mello (2000) defined anti-nutritional factors in two senses: firstly, compounds that present in human or animal foods which cause anti-nutritional effects and anti-physiological effects such as impaired reproductive function or reduced immunocompetence; secondly, substances which reduce feed intake in animals. Panhwar (2005) revealed that anti-nutritional factors also known as anti-nutrients which are poisonous substances that can be found in most food and able to limit the nutrient available to the body.

Basically, anti-nutrients can be divided into two primary groups, which are heat-stable and heat-labile group (Felix & Mello, 2000). Anti-nutrients with heat-stable property resist and can be maintained at high temperature; whereas, for anti-nutrients that fall in heat-labile group are sensitive to standard temperature and lost at high temperature (Kyriazakis & Whittemore, 2006).

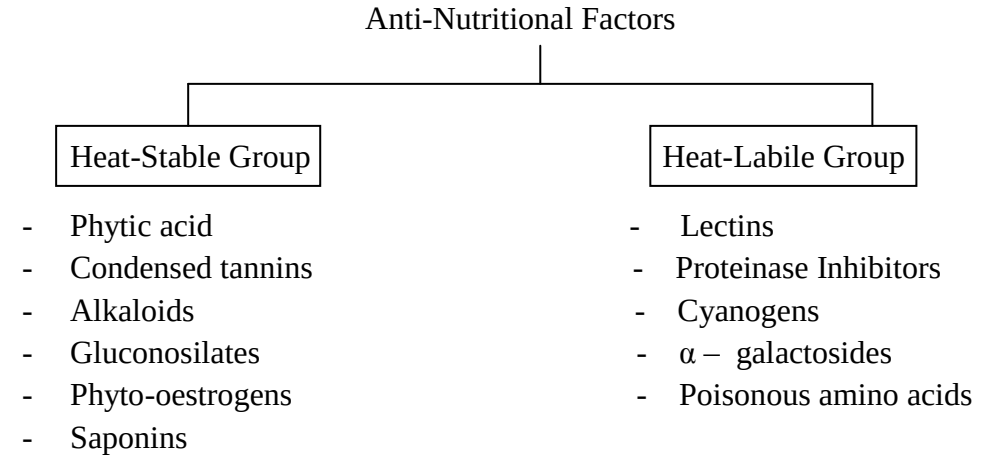


Figure 2.6: Classifications of anti-nutritional factors.

2.2.2 Relationship between anti-nutrients with bioactive compound

Anti-nutritional factors and bioactive compounds are categorized as secondary metabolites. In contrast to anti-nutrients, bioactive compounds or plant bioactives is defined as extranutritional constituents that present in certain foods and exert effects to living cells. Anti-nutrients exert anti-nutritional or anti-physiological effects on humans and animals (Felix & Mello, 2000); bioactive compounds serve as protective role for human's health condition. There are vast amounts of scientific researches which identified the protective effects of bioactive compounds in combating hypertension, cancers, cardiovascular diseases, and other health condition. The examples of bioactive compounds are phenolic compounds, flavonoids, antioxidant, hydroxytyrosol, lycopene, monoterpenes and organosulfur compounds.

Recent epidemiological and controlled-case studies reported that many anti-nutrients that present in a low level give beneficial effects for prevention of diseases like coronary diseases and cancers (Redden *et al.*, 2005). For this reason, anti-nutritional factors often known as plant bioactives or non-nutritive compounds. This implies that anti-nutrients might not always harmful even though lack of nutritive value (Muzquiz, 2000). Despite of this, the balance between beneficial and hazardous effects of plant bioactives and anti-nutrients rely on their concentration, chemical structure, time of exposure and interaction with other dietary components. Due to this, they can be considered as anti-nutritional factors with negative effects or non-nutritive compounds with positive effects on health. Non-nutritive compounds also known as pronutrients.

2.2.3 Anti-nutritional factors in peanuts

Legume family is one of the most important families in plant kingdom, in which it is a cheap protein source for vegetarian diets (Ramakrishna, Rama & Rao., 2006). Presence of anti-nutrients, such as flatulence factors, phytic acid and enzyme inhibitor limit the application of legume in food industry. There are various anti-nutrients present in different parts of peanut, such as peanut hull, skin, seed, kernel, root and cotyledons (Lindsey & Turner, 1975).

The presence of lectins in peanuts causes nonspecific interference with absorption of nutrients across intestinal wall. Lectins also known as hemagglutinin, is a plant agglutinin that exerts specific affinity for certain sugar molecules (Salunkhe, 1992). Ramakrishna *et al.* (2006) reported the presence of trypsin inhibitors inhibit the activities of protease, such as plasmin, kallikren, chymotrypsin and trypsin. Pahnwar (2005) revealed the presence of phytates, oxalates, aflatoxin, oligosaccharides and hydrogen cyanide in peanuts and their effects on health (Table 2.1).

Table 2.1: Effects of anti-nutritional compounds on body.

Anti-Nutrients	Effect on Body
Phytates	- Reduce calcium and iron absorption
Oxalates	- Reduce Calcium formation - Encourage kidney formation
Oligosaccharides	- Produce intestinal gas, discomfort and loss of appetite
Aflatoxin	- Cause liver damage and cancer
Hydrogen cyanide	- Contribute to goitre if it is low in iodine

Goitrogens is a naturally occurring compound that present in groundnut that suppresses the function of thyroid gland (Salunkhe, 1992). It interferes the uptake of iodine, which in turn causes the enlargement of thyroid gland. The phenomenon is known as “goitre”. Salunkhe (1992) also found Peanut I in groundnuts. Peanut I is a peanut protein that acts an allergic factor in inducing peanut allergy in certain individuals. The other anti-nutrients that present in groundnut include phenolic compounds, flatus agent, bitter flavour compounds and phytate.

2.3 Phytic Acid

Phytic acid (Figure 2.6) can be defined as a normal seed component with its main function as storage depot of phosphorus and used steadily during dormancy and germination (Hernández & Ortega-Delgado, 1989). Rickard and Thompson (1997) illustrate phytic acid as an anti-nutritional factor because of its ability to chelate minerals and proteins, either in direct or indirect sense, and subsequently changes its functionality, digestion, absorption and solubility.

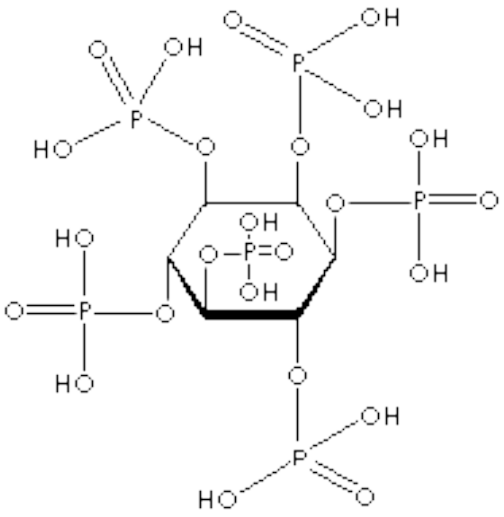


Figure 2.6: Chemical structure of phytic acid.

Phytic acid also known as 1,2,3,4,5,6-hexakis dihydrogen phosphate myoinositol, hexaphosphorylated *myo*-inositol ester, cyclohexanehexyl hexaphosphate, myo-inositol hexakisphosphate, acide fytique, acido fitico, acido fyticum and saures des phytins.

2.3.1 Distribution of phytic acid in plant kingdom

Phytic acid is widely distributed in most cereal grains, legume, oilseeds, nuts, grass family, tubers, pollen, spores and organic soil (Dost & Tokul, 2005). Makkar *et al.* (2007) reported the presence of phytic acid in *Artocarpus altilis, Cajanus cajan, Cicer arietinum, Entada scandens, Glycine max, Jatropha curcas, Phaseolus vulgaris, Sesbania sesban, Sesbania bispinosa, Treculia africana, Triticum vulgare, Vicia faba, Vigna aconitifolia, Vigna mungo, Vigna radiate* and rapeseed.

In term of distribution of phytic acid in plant tissue, phytic acid commonly detected in different organs of plants, such as seeds, grains, tubers, roots, fruits, nuts, vegetables and pollens (Roussos, 2003). Generally, 90% of phytic acid presents in the aleurone layer, while the remaining 10% localized in the embryo (Dost & Tokul, 2005). Hídvégi & Lásztity (2003) presented that aleurone grain consists of two types of inclusions, which are globoids containing different levels of phytate and protein carbohydrate bodies. Reddy *et al.* (2002) publicized that phytic acid accumulates in protein bodies or globoids and existed as a salts of minerals, like calcium (Ca), potassium (K) and magnesium (Mg). As in seeds and grains, phytic acid consists of more

than 60 – 80% of the total stored phosphorus; only 20 – 34% of the total phosphorus contains phytic acid in tubers and fruits (Morris & Ellis, 1980).

2.3.2 Biological functions of phytic acid

Liu, Hui and Zhang (2004) reported the anti-cancer property of phytic acid and show a protective action in carcinogenesis. Phytic acid acts as anti-cancer agent against colon, soft tissues, metastatic lung cancer and mammary cancer. The presence of phytic acid in dietary sources is believed as a potential antioxidant (Makkar *et al.*, 2007). It reduces iron-induced oxidative injury and reverses stimulation colorectal tumorigenesis (tumour formation) due to its mineral chelating potential. The antioxidant property of phytic acid can be used as a unique and versatile food preservative. Besides, the addition of phytic acid into fruits, vegetables, cheese, noodles, soy sauces, juices, bread, alcoholic beverages, meats, fishmeal pastes and canned seafood increase nutritive value, prolong shelf life and prevent discolouration of food (Dost & Tokul, 2006).

Phytic acid was found to reduce blood glucose and possesses health benefits to diabetic patients. The anti-nutrients concentration of phytic acid reducing the rate of starch digestion and slowing the gastric emptying, in turn lowers blood glucose. Dost and Tokul (2005) reported that phytic acid can prevent kidney stone formation. Consumption of diets rich in calcium (Ca) induces kindey calcification and subsequently renal stone development. Thus, consumption of food enriched with phytic acid able to maintain adequate Ca urinary levels to inhibit calcium oxalate crystallization. Phytic acid capable reduces cholesterol and triacylglycerol (Graf & Eaton, 1990).

2.3.3 Anti-nutritional effects of phytic acid

Literature sources stated that excessive amounts of phytic acid in the diet will form insoluble complexes with multi-charged metals (Graf, 1986; Nolan, Duffin & McWEENEY, 1987), such as copper (II), zinc (II), calcium (II) and iron (III). This results a deficit in the absorption of some dietary minerals (Erdman, 1981; Morris, 1986) and leads to mineral deficiencies. Phytic acid was reported to interact with other compounds: formation of ternary complexes of phytic acid with protein and carbohydrate (starch) will reduce their bioavailability and digestion (Makkar *et al.*, 2007). For instance, the formation of phytic acid – carbohydrate complex influences digestion rate of starch (Thompson, 1986). Phytic acid – protein complex will inhibit digestive enzymes. The elevated of phosphate level from high phytic acid diet of animals will lead to eutrophication and cause water pollution and depletion of water level (Roussos, 2003).

2.4 Saponins

Saponins can be defined as non-volatile, surface active compounds that are widely distributed in plant kingdom (Oleszek, 1990; Hostettmann & Marston, 2005). The term of Saponins derived from a soapwort plant, named (Genus Saponaria, Family Caryophyllaceae), and a latin word “Sapo”, which mean “soap”.

Saponins comprise a large family of structurally related compounds containing a steroid (Figure 2.7) or triterpernoid aglycone attached to one or more oligosaccharide moieties by glycosidic lingkage (Makkar *et al.*, 2007). The

sugar moieties (glycone) can be pentoses, uronic acid, glucose, galactose, rhamnose, methypentose, xylose and hexoses; the non-sugar portions (aglycones) may comprise of sterol, phenol, glycerol and methyl alcohol (Friedli, 1996). Hostettmann & Marston (1995) reported that the saponins can be divided into three main groups based on types of aglycones present:

i. Triterpene glycosides (triterpenoid saponozites, C_{30})

ii. Steroid glycosides (steroidal saponozites, C_{27})

~ Spirostanol saponin

~ Furastanol saponin

~ Nugatigenin saponin

~ Polipodo saponin

iii. Steroid alkaloid glycosides (glycoalkoloids)

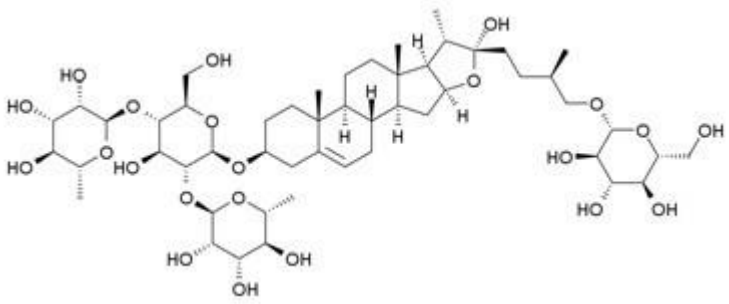


Figure 2.7: Chemical Structure of One of the Steroidal Saponins – Protodioscin.

2.4.1 Distribution of saponins in plant kingdom

Man, Gao, Zhang, Huang & Liu (2010) revealed the presence of saponins in more than 100 families of plant, as evidenced by isolation of saponins from phytochemical studies of many plant species over the years. Dini, Tenore & Gini (2009) reported in his study that saponins present widely in different plant families and also in food, such as peas, potatoes, sugar beets, asparagus, beans

and blackberries. Total steroidal saponins are mainly distributed in Amaryllidaceae, Agavaceae, Dioscoreaceae, Liliaceae, Solanaceae, Scrophulariaceae, Leguminose and Rhamnaceae (Man *et al.*, 2010). Commercial saponins are mainly extracted from dessert plants, which are *Quillaja saponaria* and *Yucca schidigera*. Within plant families, saponins was found in various parts of plant, such as leaves, stems, fruits, bulbs, blossom and roots. Dini *et al.* (2001) observed the quantitative difference of saponin contents in plant organs. Factors such as cultivar, age, geographical location and physiological state of plant determine the content of saponins.

2.4.2 Biological function of saponins

Literature sources have reported that saponins exert various biological benefits, such as anti-inflammatory, anti-diabetic, anti-HIV, anti-atherosclerotic and serve as protective functions like gastro-protective, hepatoprotective and hypolipidemic (Lee, Chung, Kim, Lee & Kim, 1995; Sawaswat, Visen, Dayal, Argawal & Patnaik, 1996; Kashiwada, Wang, Nagao, Kitanaka, Tasuda & Fujioka, 1998; Min *et al.*, 1999; Banno *et al.*, 2004). Besides, saponins are effective in maintaining liver function, lowering blood cholesterol, preventing peptic ulcer, osteoporosis as well as platelet agglutination (Kao *et al.*, 2008). The beneficial effects of saponins have been applied commercially in drugs and medicines, emulsifiers, adjuvants, taste modifiers, sweeteners and precursors of hormone synthesis (Hostettman & Marston, 1995).

2.4.3 Anti-nutritional effects of saponins

Dietary saponins highly toxic to cold-blooded animals due to its haemolytic property, in which it ruptures erythrocytes and release haemoglobin. Saponins was found to reduce nutrient utilization and conversion efficiency as in ruminants (Cheeke, 1989; Sen, Makkar & Becker, 1998). In case of monogastric animals, saponins acts as growth inhibitor and reduce their feed intake due to the bitterness and throat-irritating activity of saponins. Dietary saponins from different plants were found to reduce weight gain in salmonid fish (Bureau *et al.*, 1998), reduce fertility (Quin & Xu, 1998), and cause ruminant bloat and photosensitization (Cheeke, 1996). It affects protein digestibility by inhibit various digestive enzymes such as trypsin and chymotrypsin (Shimoyamada *et al.*, 1998; Makkar *et al.*, 2007).

2.5 Alkaloids

The general definition of "alkaloids" is the basic substances which contain one or more nitrogen atoms, usually in combination as part of a cyclic system (Harborne, 1991). In term of chemical structure, alkaloids contains one or more rings of carbon atoms with a nitrogen in the ring; the position of nitrogen is varies with different types of alkaloids in different plant families (Raffauf, 1996). Alkaloids can be classified into five major groups, which are true alkaloids, protoalkaloids, pseudoalkaloids, polyamine alkaloids and peptide and cyclopeptide alkaloids (Friedli, 1996; Robert & Wink, 1998).

2.5.1 Distribution of alkaloids in plant kingdom

Over 21,000 alkaloids are identified and isolated in plant kingdom and thus constitutes the largest group among the nitrogen-containing secondary metabolites (Fatturosso & Taglialatela, 2007). According to Robert and Wink (1998), alkaloids possess limited distribution in plant, microbial and animal species. However, the major source of alkaloids in plant kingdom is flowering plant or *Angiospermae*, in which 20% of plant alkaloids has been isolated from this. Different tissues of same plants may contain different alkaloids (Hartmann, 1991). High level of alkaloids can be determined in root, bark, fruit, seed and leaves of a plant, while other plant organs are alkaloid-free.

2.5.2 Biological function of alkaloids

Alkaloids are considered as secondary metabolite that play important role in plant interactions between animals, higher and lower plants – acting as attractant to promote pollination of plant (Robert & Wink, 1998). Alkaloids like brucine, strychnine and quinine possess high level of bitterness and thus become universal feeding deterrent in plant-herbivores interactions (Smith, 1972). Alkaloids were applied as potions, teas, poultices for hundreds of years and even serve as poison for 4000 years. It is important in food-hunting and against enemy attacks (Robert & Wink, 1998).

2.5.3 Anti-nutritional effects of alkaloids

High level of alkaloids exerts toxicity and adverse effects to humans, especially in physiological and neurological activities. For instance, consumption of tropane alkaloids will cause rapid heartbeat, paralysis and in fatal case, lead to

death. Uptake of high dose of tryptamine alkaloids will lead to staggering gate and death. However, doses differentiate between toxicity and pharmaceutical effects of alkaloids. As an illustration, lower dose of alkaloids mediate important pharmacological activities, such as analgesic, reducing blood pressure, killing tumour cells, stimulating circulation and respiration (Makkar *et al.*, 2007)

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of Peanut Root and Shells

Peanut root and shells (Figure 3.1) for this analysis was sponsored by Thong Thye Groundnut Factory Sdn. Bhd., Sungai Siput, Perak, Malaysia. Shandong peanut shell was obtained from a plantation farm in Shandong province, China. Menglembu peanut shell and peanut root came from plantation site in Vietnam. All samples were treated by removing all soil debris and dirt. Then, the samples were ground and stored in different sample vials at 4 ℃ before further analysis. This storage of samples in different vials is to ensure the moisture content of samples remain constant for each assay.

Figure 3.1: Shandong peanut shell, Menglembu peanut shell and peanut root.

3.2 Chemicals

3.2.1 Phytic acid assay based on precipitation of phytate

Sodium hydroxide, sodium sulphate, iron (III) chloride and potassium thiocyanate were purchased from System® Chemicals. Trichloroacetic acid and nitric acid were purchased from Thermo Fisher Scientific Inc. (Philadelphia, USA). Iron (III) nitrate nonanhydrate was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany). All chemicals used were analytical reagent grade.

3.2.2 Saponin assay

For preparation of saponins extract, methanol and n-hexane were purchased from QRëC™. Chloroform was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany), while n-butanol was purchased from HmbG® Reagent Chemicals (Selangor, Malaysia).

For determination of total saponins, ethanol 95% and absolute ethanol were purchased from HmbG® Reagent Chemicals (Selangor, Malaysia). Vanillin was purchased from R&M Marketing (Essex, UK). Diosgenin was purchased from Sigma Aldrich™ (St. Louis, MO, USA). Ethyl acetate was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany).

For determination of total steroidal saponins, anisaldehyde (4-methoxybenzaldehyde) and ethyl acetate were purchased from Merck Schuchardt OHG (Hohenbrunn, Germany). Sulphuric acid was purchased from

PC Laboratory Reagent. Diosgenin was purchased from Sigma Aldrich™ (St. Louis, MO, USA). All chemicals used are in analytical grade.

3.2.3 Alkaloids assay

For quantitative evaluation of alkaloids by gravimetric assay, acetic acid and ammonium hydroxide solution were purchased from HmbG® Reagent Chemicals (Selangor, Malaysia). For Qualitative evaluation of alkaloids by thin layer chromatography, acetic acid, ethanol 95%, n-butanol and ammonium hydroxide were purchased from HmbG® Reagent Chemicals (Selangor, Malaysia). Chloroform and platinum chloride was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany). Hydrochloric acid was purchased from Thermo Fisher Scientific Inc. (Philadelphia, USA). Methanol was purchased from QR ë C™, while potassium iodide was purchased from System® Chemicals. All chemicals were graded as analytical reagent grade.

3.3 Determination of Moisture Content

Refer to method of Bradley, R.L. (1994), 5 g of sample was placed into moisture dish, the mass of moisture dish with sample was measured. The moisture dish was placed into oven (Modell UNE 500 PA; Memmert, Germany) at 105℃ for 5 hours. After 5-hours incubation, the moisture dish was transferred to desiccators for cooling purpose. The new mass of moisture dish with sample was measured using analytical balance (Model CP224S; Sartorius AG, Goettingen, Germany).

The moisture dish with sample was then incubated in the oven at 105℃. The mass of moisture dish with sample was taken every one hour consecutively until the changes of mass become constant. Weight changes in the oven drying method are assumed to be due to moisture loss. The moisture content can be calculated by applying the formula as below:

$$\% \text{ Moisture (wt/wt)} = \frac{\text{wt of wet sample} - \text{wt of dry sample}}{\text{wt of wet sample}} \times 100$$

3.4 Phytic Acid Assay based on Precipitation of Phytate

According to procedure of Wheeler and Ferrel (1971) for preparation of Iron (III) nitrate nonanhydrate calibration curve as well as preparation of samples prior to spectrophotometric assay, modification has been made on dilution factor in order to obtain the absorbance values that obey Beer-Lambert Law.

3.4.1 Preparation of iron (III) nitrate nonanhydrate calibration curve

Stock standard solution was prepared by mixing iron (III) nitrate nonanhydrate (4.33 mg/mL, 72.89 g) into 100 mL of distilled water. 25 times dilution was conducted by taking 2 mL of the stock standard solution and made up to 50 mL in a volumetric flask with distilled water. 2.5 mL, 5.0 mL, 7.5 mL, 10.0 mL and 12.5 mL aliquots were taken from this working standard into a series of 25 mL volumetric flask. Potassium thiocyanate (1.5 M, 5 mL) was added and the volume was made up to 25 mL with distilled water.

3.4.2 Extraction and determination of phytic acid

In order to obtain uniform particle size of ground sample for extraction, the sample was sieved to 35-mesh by using a 0.5 mm sieve. 10 g of finely ground

35-mesh sample which contains 5 – 30 mg phytate – P by estimation was weighed and placed into a 250 mL of Erlenmeyer flask. The phytate was extracted in TCA (3%, 50 mL) by mixing on a magnetic stirrer (LMS-1003, Daihan Labtech Co. Ltd, Korea) for 30 mins.

The suspension was centrifuged using centrifuge (Model 5430; Eppendorf, New York, USA) at 3000 g for 10 mins. A 10 mL aliquot of supernatant was transferred to a 50 mL conical centrifuge tube. $FeCl_3$ solution (5.83 mg/mL, 4 mL) was added rapidly to the aliquot of centrifuge tubes. The content was heated in a boiling water bath (Oilbath ONE; Memmert, Germany) for 45 mins. One to two drops of 3% Sodium sulphate in 3% TCA was added if the supernatant was not clear after 30 mins. The heating process was then continued.

The suspension was centrifuged (Model 5430; Eppendorf, New York, USA) at 3000 g for 10 – 15 mins and the clear supernatant was decanted. The precipitate was washed twice by dispersing it well in TCA (3%, 20 – 25 mL). The suspension was heated in a boiling water bath (Oilbath ONE; Memmert, Germany) for 5 to 10 mins and then centrifuged (Model 5430; Eppendorf, New York, USA) at $3000g$ for 10 mins. The washing of precipitate was repeated with distilled water.

The precipitate was dispersed in a few mililitres of distilled water and sodium hydroxide (1.5 M, 3 mL) was added with mixing. The volume was brought to approximately 30 mL with distilled water and was heated in boiling water bath

(Oilbath ONE; Memmert, Germany) for 30 mins. The suspension was filtered hot (quantitatively) through moderately retentive paper (Filter Paper No. 389; Sartorius, Goettingen, Germany). The precipitate was washed with 60 to 70 mL of hot distilled water and the filtrate was discarded. The precipitate on the filter paper was transferred and dissolved into the 25 mL volumetric flask containing nitric acid (3.2 N, 10 mL). The filter paper was washed with several portions of distilled water and the filtrate was collected in the same flask. The flask and contents was cooled to room temperature and the volume was brought to 25 mL with distilled water.

A 5 mL aliquot was transfer to another 25 mL volumetric flask. Potassium thiocyanate (1.5 M, 20 mL) was added and the volume was brought to 25 mL with distilled water. The colour of solution was read immediately (within1 min) at 480 nm using a spectrophotometer (Genesys 10 UV; Thermo Fisher Scientific Inc., Philadelphia, USA). The reagent blank was run with each set of samples. Based on the calibration curve, the amounts of iron present (µg) was determined and the phytate-P was calculated from the equation as below:

$$\frac{\text{Phytate-P } (mg)}{100g \text{ Sample}} = \frac{\text{Fe } (\mu g) \times 15}{\text{Weight of Sample } (g)}$$

3.5 Qualitative and Quantitative Determination of Saponins

According to Baccou,Lambert & Sauvaire's procedure (1977), determination of total steroidal saponins in peanut by-products can be carried out by spectrophotometric assay. Refer to Hiai, Oura and Nkajima (1976) for spectrophotometric determination of total saponins, the amounts of diosgenin

in each aliquot was modified in order to obtain an ideal linear regression in calibration curve of total saponins.

3.5.1 Detection of saponins through foam test

Acoording to procedure of Domingo et al. (2009), soap-forming property of saponins can be used as a marker to detect the presence of saponins in peanut by-products. Approximately 2 g of ground sample was boiled in 20 mL of distilled water in a boiling water bath (Oilbath ONE; Memmert, Germany). Filtration was conducted using filter paper (Filter Paper No. 389; Sartorius, Goettingen, Germany) after boiling process. 5 mL of distilled water was added to 10 mL of filtrate. The mixture was shaken vigorously for formation of a stable persistent froth. 3 drops of olive oil was added into the froth and continues shake vigorously. The positive result indicated with the formation of emulsion.

3.5.2 Detection of saponins through froth test

The presence of saponins can be detected qualitatively by the formation of honeycomb froth with its height greater than 2 cm (Domingo et al., 2009). 1 g of ground sample was weighed into a conical flask in which 10 mL of sterile distilled water was added and boiled for 5 mins in a boiling water bath (Oilbath ONE; Memmert, Germany). The mixture was filtered using filter paper (Filter Paper No. 389; Sartorius, Goettingen, Germany). 2.5 mL of filtrate was added to 10 mL of sterile distilled water in a test tube. The tube was stopped and shaken vigorously for about 30 seconds. Few drops of 5% sodium carbonate solution can be added to basify the extract if the plant extract

with poor frothing effect. The tubes were then allowed to stand for half an hour. The formation of honeycomb froth was observed.

3.5.3 Preparation of saponins extract

The samples were defatted according to the method of Sanbongi *et al.* (1998) with some modifications. Ten g of finely ground 35-mesh samples were soaked with n-hexane in a ratio of 1:5. The suspension was stirred for 45 mins at ambient temperature. The mixture was left overnight at room temperature and filtration was conducted by using moderately retentive filter paper (Filter Paper No. 389; Sartorius, Goettingen, Germany). The residue remaining was defatted again after filtration. The defatted samples were dried overnight in a fume hood at room temperature. Subsequently, the defatted samples were dried in a 40 ℃ air-oven (Modell UNE 500 PA; Memmert, Germany) for 1 hour to remove n-hexane residue.

Ten g of defatted sample was taken into a 250-mL Erlenmeyer flask and aqueous methanol was added (50%, 100 mL). The suspension was stirred on a magnetic stirrer for overnight at room temperature. The contents were centrifuged (Model 5430; Eppendorf, New York, USA) at 3000 g for 10 mins and the supernatant was collected. The extraction with the same solvent was repeated by stirring on a magnetic stirrer for overnight. The first supernatant was combined with the second one after centrifugation. Filtration was conducted using filter paper (Filter Paper No. 389; Sartorius, Goettingen, Germany) if needed.

The methanol was evaporated from the solution under vacuum at approximately 42℃ by using a rotary evaporator (Laborata 4000; Heidolph Instruments GmbH and Co. KG, Germany). The aqueous phase was centrifuged (Model 5430; Eppendorf, New York, USA) at 3000 g for 10 mins to remove the insoluble materials. The aqueous phase was transferred into a separating funnel and equal volume of chloroform (three times) to remove pigments. The concentrated saponins were extracted in the aqueous solution with equal volume of n-butanol (two times). The solvent n-butanol was evaporated under vacuum at a temperature not higher than 45℃. The dried fraction containing saponin was dissolved in 5 – 10 mL of distilled water and the solution was transferred into a separate vial and freeze-dried (Scanvac CoolSafe 55-9; Denmark). The percent recovery of saponins was calculated.

3.5.4 Determination of total saponins

To prepare standard curve, 100, 125, 150, 175, 200, 225 and 250 µL of the standard saponin solution were placed into test tubes and the volumes were made up to aqueous methanol (80%, 0.25 mL). Standard saponin solution prepared by dissolve 10 mg of diosgenin in mixture of methanol (16 mL) and distilled water (4 mL). To the aliquots for each tube, vanillin reagent (8%, 0.25 mL) was added and sulphuric acid (72% v/v, 2.5 mL) added slowly on the inner side of the wall. The solutions were mixed well and the tubes were transferred to a 60^{o}C water bath (Oilbath ONE; Memmert, Germany). After 10 mins incubation, the tubes were cool in ice-cold water bath for 3 – 4 mins. The absorbance was measured at 544 nm Genesys 10 UV; Thermo Fisher Scientific Inc., Philadelphia, USA) against the reagent blank. 0.1 g of freeze-dried sample

was dissolved in aqueous methanol (80%, 0.1 mL). 0.25 mL of aliquot was taken for spectrophotometric determination for total saponins at 544 nm.

3.5.5 Determination of total steroidal saponins

To prepare standard curve, 0, 20, 40, 60, 80 and 100 µL of the diosgenin standard solution (0.1 mg/mL) were placed in test tubes and the volume was made up to 2 mL with ethyl acetate. Disogenin standard solution was prepared by dissolve 10 mg of disogenin in ethyl acetate (99.5%, 100 mL). 1 mL of reagent A and 1 mL of reagent B were added. Reagent A was prepared by mixing anisaldehyde (0.5 mL) and ethyl acetate (99.5%, 99.5 mL); Reagent B is the mixture of concentrated sulphuric acid (95 – 98% w/w, 50 mL) and ethyl acetate (99.5%, 50 mL). After stirring, the test tubes were placed at room temperature for 30 mins. The absorbance was measured at 430 nm using UV-visible spectrophotometer (Genesys 10 UV; Thermo Fisher Scientific Inc., Philadelphia, USA) against the reagent blank.

For sample determination, a known amount of freeze-dried crude saponins was dissolved in aqueous methanol (80%, 1 mL). The suitable aliquots contain 0 – 40 µg sapogenin by estimation were taken into test tubes. The test tubes were placed in a boiling water bath (Oilbath ONE; Memmert, Germany) for removal of alcohol. After cooling, ethyl acetate (99.5%, 2 mL) was added into test tubes and the spectrophotometric determination was conducted at 430 nm against the reagent blank.

3.6 Alkaloids Assay

3.6.1 Qualitative evaluation of alkaloids by gravimetric assay

The gravimetric determination of alkaloids follows procedure which proposed by Harbone (1973) and further explained by Onwuka (2006). Five g of ground sample was measured using an analytical balance (CP334S; Sartorius, Goettingen, Germany) and dispersed in 50 mL of 10% acetic acid in methanol. The suspension was shaken in shaking incubator (Daihan Labtech Co. Ltd, Korea) at 120 rpm and 30 ℃. The suspension was allowed to stand for 4 hours before filtration. Filtration was conducted using filter paper (Filter Paper No. 389; Sartorius, Goettingen, Germany) and the filtrate was evaporated to ¼ of its original volume using hotplate (LMS-1003, Daihan Labtech Co. Ltd, Korea). Concentrated ammonium hydroxide (30%) was added drop-by-drop to precipitate alkaloids from the filtrate. A pre-weighed filter paper (Filter Paper No. 389; Sartorius, Goettingen, Germany) was used to filter off the precipitate and it was then washed with ammonium hydroxide solution (1%). The filter paper with alkaloids precipitates was dried in 60 ℃ oven for 30 mins (Modell UNE 500 PA; Memmert, Germany). The filter paper was transferred from oven to dessicator for cooling purpose. The filter paper with dried alkaloids was reweighed until a constant weight was obtained. The content of alkaloids is determined by the weight difference between of the filter paper and expressed as percentage.

3.6.2 Identification of alkaloids classes by thin layer chromatography

Eight g of sample was extracted in acetic acid (10%, 40 mL) in methanol. The mixture was kept for 4 hours under dark condition on shaking incubator

(Daihan Labtech Co. Ltd, Korea) at 120 rpm and 30 ℃ at room temperature and the contents was centrifuged (Model 5430; Eppendorf, New York, USA) at 7000 g for 50 mins. The extract was concentrated to one quarter of the original volume using rotary evaporator (Laborata 4000; Heidolph Instruments GmbH and Co. KG, Germany) and the alkaloids were precipitated by drop-by-drop addition of concentrated ammonium hydroxide (30%). The precipitate was collected by centrifugation at 3000 g for 10 mins using centrifuge (Model 5430; Eppendorf, New York, USA) and washed with ammonium hydroxide solution (1%). Due to no formation of precipitate, the alkaloids were extracted three times with chloroform, 40 mL of each time. The volume of alkaloid extract was reduced by using a rotary evaporator at 35 ℃ to about 2 – 4 mL and kept for further analysis.

An aliquot of extract (5 – 10 µL) was applied on the silica gel plates. The plate was placed in tank with developing solvent mixture of methanol/ concentrated ammonium hydroxide. The tank was equilibrated for at least 1 hour with the developing solvent before the TLC plate was inserted. The presence of alkaloids on the developed plate was detected by observing fluorescence under ultraviolet light (UV) spectrum. The position of spot was marked and Retention Factor, R_f value was calculated as below:

$$R_f = \frac{\text{Distance traveled by the center of the zone}}{\text{Distance traveled by the developer front}}$$

CHAPTER 4

RESULTS

4.1 Moisture Content of Peanut Shell and Peanut Root

Moisture content in food samples expressed as percentage. Refer to Table 4.1, the moisture content of Shandong peanut shell and Menglembu peanut shell is 9.56% ± 0.00 and 9.50% ± 0.00 whereas peanut root has moisture content as high as 9.94% ± 0.00. The difference of moisture content between Shandong peanut shell and Menglembu peanut shell is 0.06%.

Table 4.1: The moisture content and coefficient of variation of Shandong peanut shell, Menglembu peanut shell and Peanut root.

Parameter / Samples	Moisture Content (%)	Coefficient of Variation (%)
Shandong Peanut Shell	9.56 ± 0.00	0.04
Menglembu Peanut Shell	9.50 ± 0.00	0.05
Peanut Root	9.94 ± 0.00	0.05

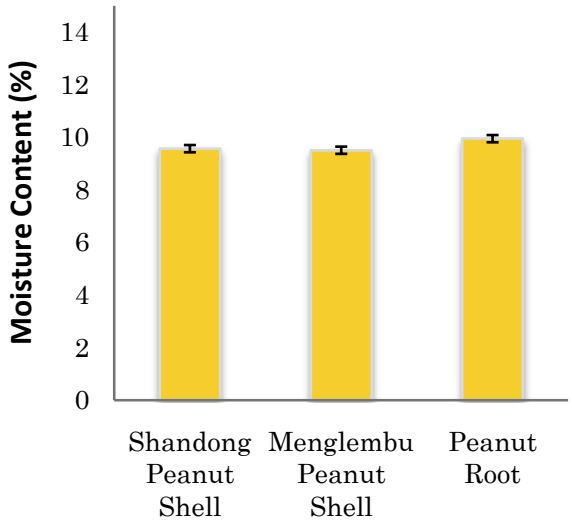


Figure 4.1: Moisture content of Shandong peanut shell, Menglembu Peanut shell and peanut root.

4.2 Phytic Acid Content of Peanut Shell and Peanut Root

By referring to Figure 4.1, it shows the calibration curve of phytic acid assay based on precipitate of phytate. The regression coefficient is 0.978 and this indicates that the absorbance values obtained in constructing the calibration curve has a high reliability and accuracy. The quantification of phytic acid content (Table 4.2) from absorbance values was calculated based on formula. Shandong peanut shell has the highest phytic acid content among other samples, which is 0.165 ± 0.000 g/ 100 g.

Table 4.2: The amount of Phytic acid (g/100 g) of Shandong peanut shell, Menglembu peanut shell and Peanut root.

Parameter / Samples	Absorbance Value	Amount of Phytic Acid (g/ 100g)	Coefficient of Variation (%)
Shandong Peanut Shell	0.109	0.165 ± 0.000	0.53
Menglembu Peanut Shell	0.071	0.107 ± 0.000	0.81
Peanut Root	0.107	0.161 ± 0.000	0.55

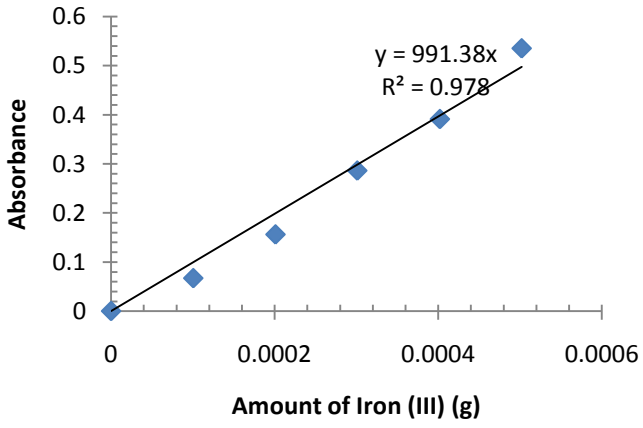


Figure 4.2: Calibration curve of iron (III) nitrate nonanhydrate.

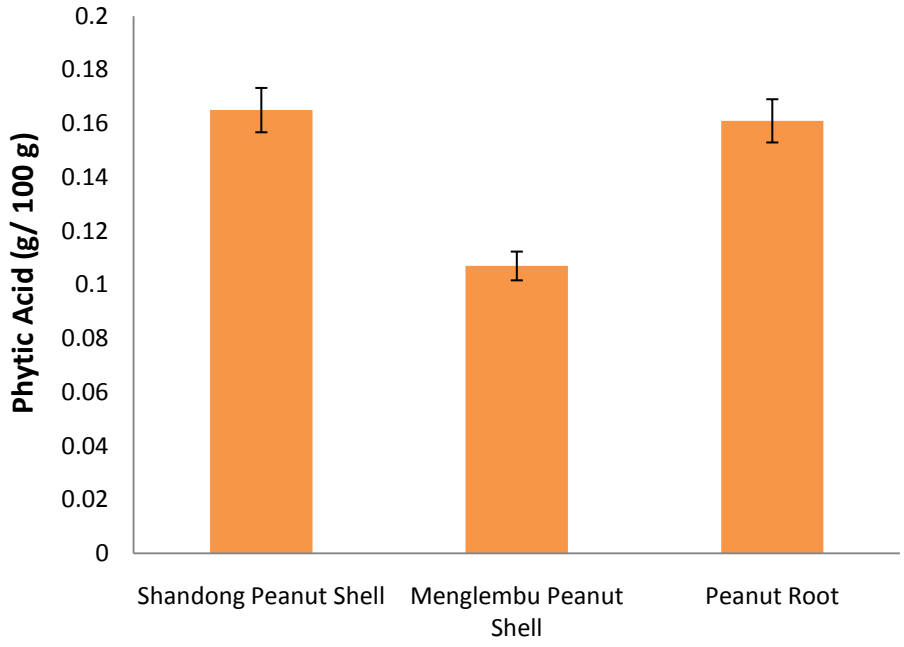


Figure 4.3: Phytic acid content of Shandong peanut shell, Menglembu peanut shell and peanut root.

4.3 Saponins Content of Peanut Shell and Peanut Root

4.3.1 Detection of saponins through foam test

The soap-forming property is a qualitative marker for presence of saponins in food sample. Refer to Figure 4.4(a), it shows the foam formation in Shandong peanut shell, Menglembu peanut shell and peanut root just after vigorous shaking process and addition of olive oil. Figure 4.4(b) shows the foam formation in sample tubes after 30 minutes at room temperature. In comparison, the foam is white colour in Figure 4.4(a), while Figure 4.4(b) appeared translucent rather than whitish in colour.

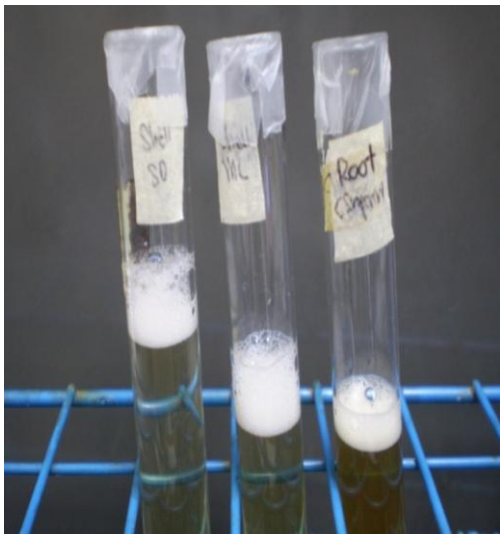


Figure 4.4(a): The soap emulsion of Shandong peanut shell, Menglembu peanut shell and peanut root after vigorous shaking process.

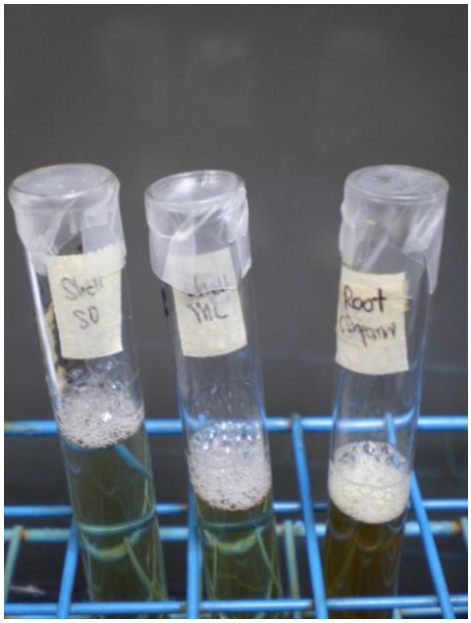


Figure 4.4(b): The soap emulsion of Shandong peanut shell, Menglembu peanut shell and peanut root after 30 minutes.

4.3.2 Detection of saponins through froth test

Formation of honeycomb froth with its height greater than 2 cm indicates positive result for froth test. Based on Figure 4.5 (a), (b) and (c), it shows the formation of honeycomb froth of peanut root, Shandong peanut shell and

Menglembu peanut shell. Refer to table 4.6(a), Menglembu peanut shell and peanut root has same height of honeycomb froth, which is 2.3 cm. In contrast, the height of honeycomb froth of Shandong peanut shell is only 2.0 cm.

Table 4.3: The height of honeycomb froth in sample tubes of Shandong peanut shell, Menglembu peanut shell and peanut root.

Sample	Height of Honeycomb froth (cm)	Coefficient of Variation (%)
Shandong	2.0 ± 0.0	3.0
Menglembu	2.3 ± 0.0	2.6
Root	2.3 ± 0.0	0.0

Figure 4.5(a): The honeycomb Froth Formation of Shandong Peanut Shell.

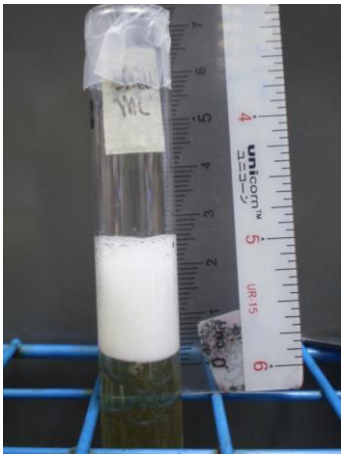


Figure 4.5(b): The Honeycomb Froth Formation of Menglembu Peanut Shell.

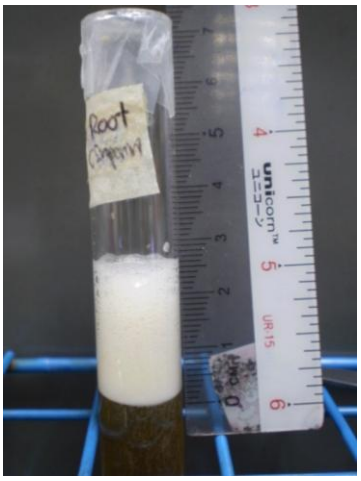


Figure 4.5(c): The honeycomb froth formation of peanut root.

4.3.3 Percent recovery of saponins extract

Percent recovery can be calculated by applied formula as below:

$$\text{Percent Recovery (\%)} = \frac{\text{Mass of samples after freeze-drying (g)}}{\text{Mass of samples before freeze-drying (g)}} \times 100$$

Table 4.4: Percent recovery (%) of Shandong peanut shell, Menglembu peanut shell and peanut root.

Sample / Parameter	Shandong Peanut Shell	Menglembu Peanut Shell	Peanut Root
Weight of Freeze-dryer flask (g)	268.95	268.95	219.19
Weight of Freeze-dryer flask + Weight of Sample before freeze drying (g)	276.95	278.25	228.69
Weight of Freeze-dryer flask + Weight of Sample after freeze drying (g)	269.83	270.00	220.29
Percent Recovery (%)	97.43	97.04	96.33
Weight of freeze-dried Saponin (g)	0.88	1.05	1.10

4.3.4 Determination of Total Saponins

Figure 4.6 shows calibration curve of total saponins with regression coefficient of 0.9592. Table 4.5 shows the total saponins content of samples with their absorbance values and coefficient of variation. Shandong peanut shell has the lowest absorbance value as well as total saponins content, which are 0.429 and m12.30 ±0.03 mg/ 10 g. In contrast, total saponins content of Shandong peanut shell and peanut root are closely approximate to each other. Peanut root has the highest level of total saponins that is 21.20 ±0.02 mg/ 10 g. The coefficients of variation for all samples are very low in Shandong peanut shell (0.27 %), Menglembu peanut shell (0.14 %) and peanut root (0.08 %).

Table 4.5: Total Saponins Content of Shandong Peanut Shell, Menglembu Peanut Shell and Peanut Root.

Parameter / Samples	Absorbance Value	Amount of Total Saponins (mg/ 10g)	Coefficient of Variation (%)
Shandong Peanut Shell	0.429	12.30 ±0.03	0.27
Menglembu Peanut Shell	0.701	20.00 ±0.03	0.14
Peanut Root	0.743	21.20 ±0.02	0.08

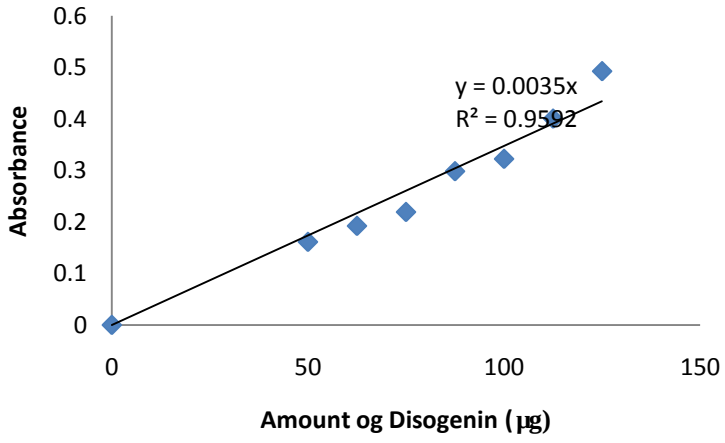


Figure 4.6: Calibration curve of total saponins.

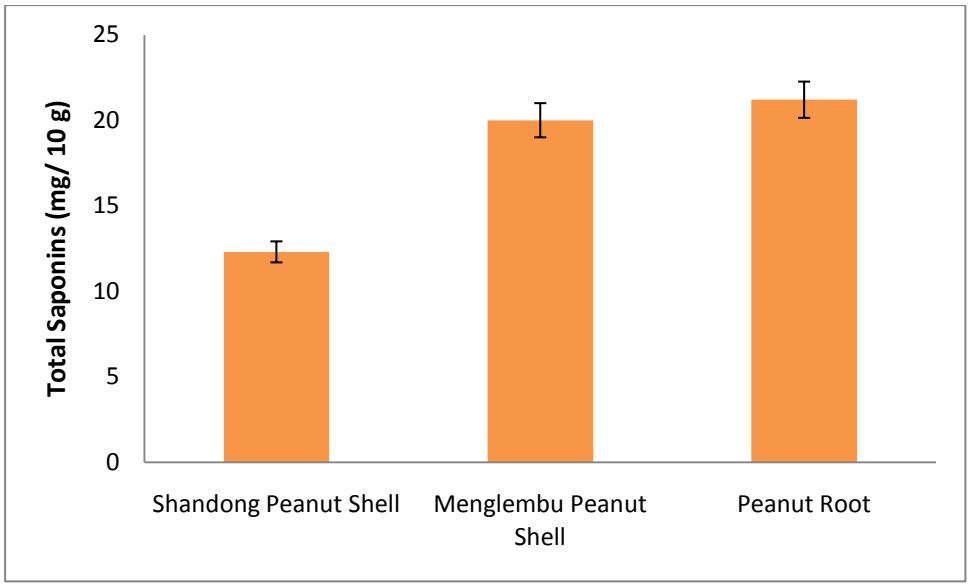


Figure 4.7: Total saponins of Shandong peanut shell, Menglembu peanut shell and peanut root.

4.3.5 Determination of Total Steroidal Saponins

Table 4.6 shows the total steroidal saponins content of samples with their absorbance value and coefficient of variation. Shandong peanut shell possesses the lowest amount of total steroidal saponins, which is 0.90 ± 0.00 mg/ 10 g. Among three samples, peanut root has the highest level of total steroidal saponins, which is 2.57 ± 0.00 mg/ 10 g and followed by Menglembu peanut shell with 2.50 ± 0.00 mg/ 10 g. The range of coefficient of variation is 0.07 – 0.48 %.

Table 4.6: Total steroidal saponins of Shandong peanut shell, Menglembu peanut shell and peanut root and their absorbance values.

Parameter / Samples	Absorbance Value	Amount of Total Steroidal Saponins (mg/ 10g)	Coefficient of Variation (%)
Shandong Peanut Shell	0.316	0.90 ± 0.00	0.48
Menglembu Peanut Shell	0.880	2.50 ± 0.00	0.11
Peanut Root	0.904	2.57 ± 0.00	0.07

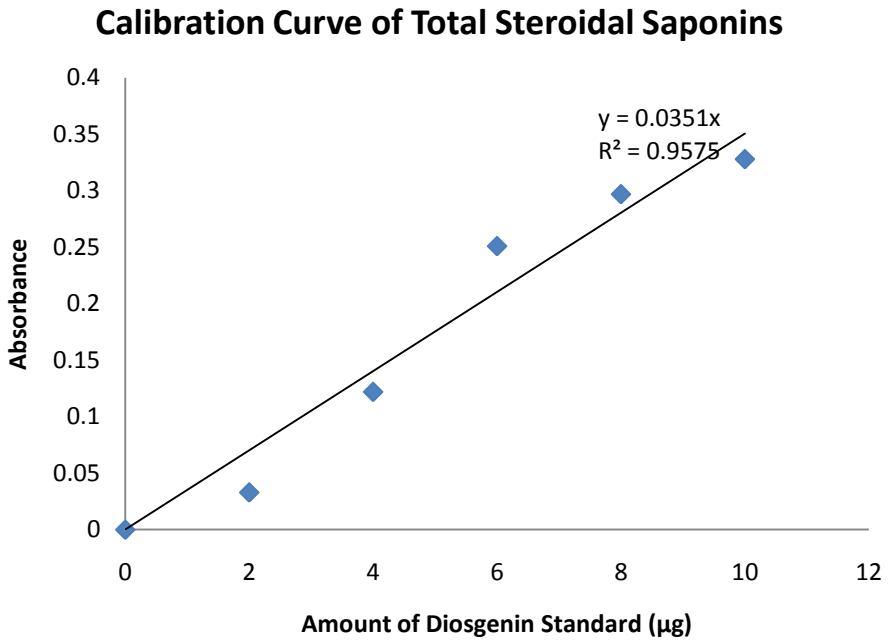


Figure 4.8: Calibration curve of total steroidal saponins.

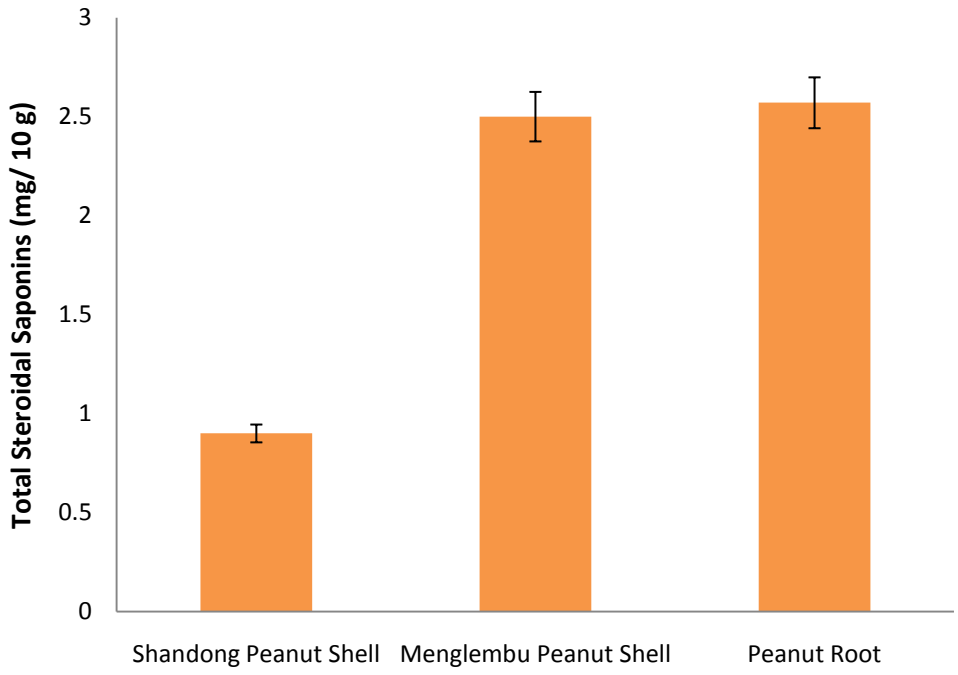


Figure 4.9: Total steroidal saponins of Shandong peanut shell, Menglembu peanut shell and peanut root.

4.4 Alkaloids Assay

4.4.1 Quantitative Evaluation of Alkaloids by Gravimetric Assay

By conducting gravimetric assay, the amount of alkaloids can be quantified by the weight differences of alkaloids precipitate before and after drying. Refer to Table 4.7, it clearly shows that percentage of alkaloids precipitate of peanut by-products in range of 4 – 24%. Peanut root has the highest percentage of alkaloids precipitate, which is 23.77 % ± 0.02. In contrast, Shandong peanut accounts lowest alkaloids precipitate that is 4.46 % ± 0.01.

Table 4.7: The Percentage of Alkaloids Precipitate in Shandong Peanut Shell, Menglembu Peanut Shell and Peanut Root.

Parameter / Samples	Percentage of Alkaloids Precipitate (%)	Coefficient of Variation (%)
Shandong Peanut Shell	4.46 ± 0.01	0.22
Menglembu Peanut Shell	8.35 ± 0.01	0.12
Peanut Root	23.77 ± 0.02	0.08

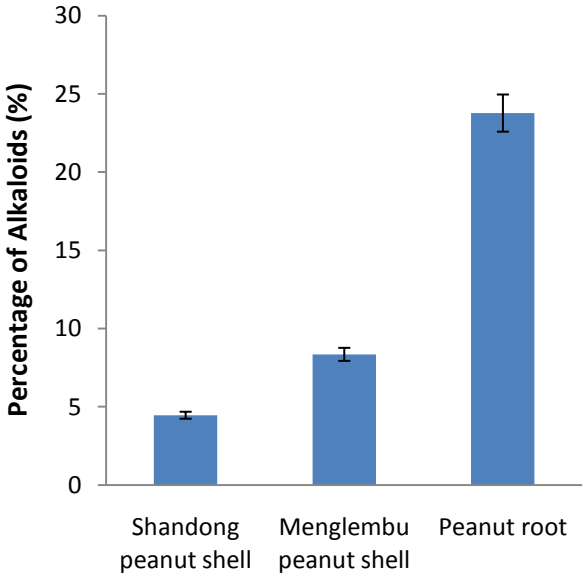
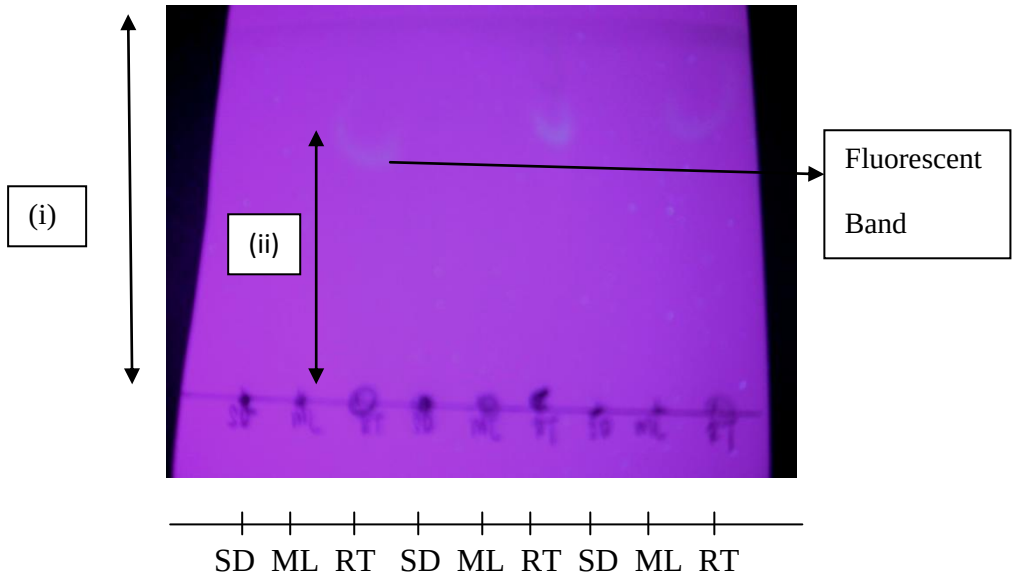


Figure 4.8: Percent of alkaloids in peanut root and shells.

4.4.2 Identification of Alkaloids Classes by Thin Layer Chromatography

Figure 4.9 shows the fluorescent image of TLC plate under ultraviolet light. For Shandong and Menglembu peanut shells, there are no any bands can be observed under ultraviolet light which indicates the absence of alkaloids in these samples. In contrast, fluorescent yellow bands can be observed in peanut root under ultraviolet light. According to Table 4.8, average value of R_f is 0.65 ± 0.02. This indicates the presence of class Berberine alkaloids in peanut root as its retention factor closely approximate to 0.70 (Clarke, 1970).



Legend: SD – Shandong Peanut Shell

ML – Menglembu Peanut Shell

RT – Peanut Root

$$R_f = \frac{\text{Distance traveled by the center of the zone, (ii)}}{\text{Distance traveled by the developer front, (i)}}$$

Figure 4.9: The presence of alkaloids indicated by fluorescent bands in thin layer chromatography.

Table 4.7: The retention factor values of Shandong peanut shell, Menglembu peanut shell and peanut root.

Sample	Retention Factor, R_f			Average
	1	2	3	
Shandong Peanut Shell	-	-	-	-
Menglembu Peanut Shell	-	-	-	-
Peanut Root	0.63	0.66	0.67	0.63 ± 0.02

CHAPTER 5

DISCUSSION

5.1 Moisture Content of Peanut Shell and Peanut Root

Based on the experimental result, the moisture content for Shandong peanut shell, Menglembu peanut shell and peanut root is 9.56% ± 0.00, 9.50% ± 0.00 and 9.94% ± 0.00. Moisture content for all samples is below 12%, and this fits the theoretical range of moisture content for all low moisture food (Bradley, 1994). Peanut root has the highest moisture content among three samples. This probably correlates with primary function of root system, which absorbs water from soil and thus retain the highest water content compare with peanut shells (Parker, 2009; Biles, 2010). In terms of peanut shell, there is not much significance of moisture content between Shandong peanut shell and Menglembu peanut shell. This implies that peanut shell contains the same moisture content regarding different types and cultivars.

Moisture content of these samples not exceed 75% are suitable to be utilized as feedstuff for swine and poultry (Crickenberger & Carawan, 1996). Peanut shell and peanut root can be classified as low moisture biological materials, therefore it is safe from microbiological spoilage and possible aflatoxin production by mould. Due to this, it can be eliminate the need for exhausting drying process and reduce cost in manufacturing process. Thus, moisture content of by-products is an important factor determines whether it is economical to be utilized as animal feedstuffs. In addition, determination of

moisture content in samples is important to ensure that the result obtained from the other succeeding experiment is normalized on dry weight, thus eliminating the differences in moisture content factor.

5.2 Phytic Acid Content of Peanut Shell and Peanut Root

Shandong peanut shell has the highest phytic acid content, which is 0.165 ± 0.00 g/ 100 g and closely followed by peanut root with 0.161 ± 0.00 g/ 100 g. In contrast, the phytic acid content of Menglembu peanut shell is significantly lower than the other samples, which are only 0.107 ± 0.00 g/ 100 g. Low percentage of correlation of variation ensure the precision and reliability of triplicate readings for each sample analysis. Although there is none scientific study on phytic acid in peanut root and peanut shell, the experimental phytic acid level was compared with phytic acid content of peanut seeds or other agricultural by-products reported in previous studies.

Phytic acid level varies in different organs of plants, such as seeds, grains, roots, tuber, fruits, nuts and pollens. The amount of phytic acid in other organs of a plant is relatively lower than in seed and grains (Maheswari *et al.*, 2010). According to McCance & Widdowsons (2010), the range of phytic acid in peanut seeds or nuts is 0.290 g/ 100 g – 0.770 g/ 100 g. Therefore, it can be assumed than the phytic acid content in organs other than seeds, such as peanut root and shell is lower than this range. The range of phytic acid amount in peanut root and shell which obtained from experiment is 0.107 – 0.165 g/ 100 g, thus fits the theoretical assumption.

Besides, phytic acid level in different organs of plant can be correlated with the amount of proteins and minerals. Phytic acid level was more predominant in seeds of plant due to its high protein content. In contrast, protein level is less abundant in peanut root and shell and thus their phytic acid level is lower than in seeds. Protein molecule with charged-side chains are able to interact with phytic acid and form phytic acid – protein complexes (Hídvégi & Lásztity, 2003). Mineral content in plant material also influence its phytic acid level. Phytic acid possess negative charge at wide range of pH, except pH that corresponds to isoelectric point, pI; its negative charge interacts with positive charge from multivalent cations, such as Zn^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+} and Fe^{3+} (Hídvégi & Lásztity , 2003; Roussos, 2003; Makkar *et al.*, 2007). Thus, wherever these kinds of minerals present in different part of organs, formation of phytic acid – mineral complexes will be induced due to the interaction between each others. The negative effect of phytic acid to bioavailability of minerals has been reported in numerous previous reports. It can deduced that the higher the phytic acid, the greater amount of phytic acid – mineral complexes can be formed, and thus the lower bioavailability of minerals to living organisms.

Doses of phytic acid differentiates it beneficial and hazardous effect to living organisms. Phytic acid can be considered as bioactive compound by exerting beneficial effects or anti-nutritional compounds with hazardous effect (Muzquiz *et al.*, 2000). Previous studies reported that phytate level in animal feedstuffs that exceed the concentration of 0.5% was detrimental to animals (Francis, Makkar & Becker, 2001). The concentration of phytic acid can also

be expressed in percent phytate – grams of phytate per 100 g of peanut root and shells. By conducting this conversion, phytic acid level in Shandong peanut shell, Menglembu peanut shell and peanut root is 0.17%, 0.11% and 0.16%. Hence, the phytic acid level in these samples does not exceed the toxicity threshold, which is 0.5%. This implies that the concentration of phytic acid level in peanut root and shells is not able to exert anti-nutritional effects, such as reduce bioavailability of minerals and protein to livestocks if it applied as animal feedstuffs.

However, numerous of physical, chemical and enzymatic methods can be utilized to reduce the phytic acid level in peanut by-products before further processing into animal feedstuffs. The processing techniques include heat treatment (cooking, baking, autoclaving and extrusion), soaking, germination, dehulling, alkaline treatment and action of phytate degrading enzymes were found to reduce phytic acid content in food samples and increase minerals absorption in living organisms (Hídvégi & Lásztity, 2003).

5.3 Saponins Content of Peanut Shell and Peanut Root

Extraction step is the most important stage for isolation, recovery and purification of anti-nutritional factors, such as saponins from plant materials. Due to relatively large molecular weight and high polarity of saponins, there are numbers of challenges lies in extraction procedure. Determination of total steroidal saponins and total saponins share the same protocol of extraction. Prior to extraction, samples were undergone defatting procedure using hexane

to remove lipids and pigments that might interfere saponins analysis (Felix, Mello & Duffus, 1991).

50% aqueous methanol chosen as the extraction solvent as it provide a high yield of saponins content compare to others, such as glycerol/water (65:35; v/v) and ethanol/glycerol/water (20:40:40; v/v/v) as proposed by Kao *et al.* (2008). The purpose of using methanol to extract defatted residues is to isolate saponins with low molecular weight species, which are sugars, phenolics, glycosides, flavonoids and oligosaccharides out from samples. The use of rotary evaporator after methanol extraction is to evaporate and remove methanol from sample mixture. Extraction of saponins is followed by using n-butanol, this process resembles liquid-liquid extraction. Precaution must be taken to control well temperature of rotary evaporator. This is because hot extraction will lead to formation of artifacts due to disintegration of labile substances like esters and acylated –formed substances (Felix *et al.*, 1991). Freeze-drying is important to preserve extracted saponins as well as increase their shelf life in a process of analysis and determination. Besides, the weight of extracted saponins before and after freeze-drying process is important in performing calculation of percent recovery for each sample.

5.3.1 Detection of saponins through foam test

Foam test is a simple and general qualitative test that determines the presence of saponins in peanut root and shells. It is usually used as first indicator of the presence of saponins in plant extracts (Cheeke, 1989). The principal of assay is developed based on general characteristic of natural foaming property or

biosurfactant activity of saponins. Refer to Figure 4.4 (a) and (b), it is clearly shows formation of soap-like foam in sample tubes of Shandong peanut shell, Menglembu peanut shell and peanut root. The foaming property of saponins is due to interaction of hydrophobic (fat-soluble) sapogenin with hydrophilic (water-soluble) sugar parts of saponins during agitation (Domingo *et al.*, 2009). The soapy character of saponins plant extract offers a wide range of benefits to humans and living organisms. For instance, saponins plant extracts important in ore separation in industrial and mining operation, and usually use as foaming agents in beverages such as root beer as well as useful in products, like cosmetics, shampoo and photographic emulsions. Saponins plant extracts also used to produce natural detergent or soap. It is advantageous than synthetic detergents as it can cure eczema or hypersensitivity type IV.

5.3.2 Detection of saponins through froth test

Froth test shares few similarities with Foam test which as mentioned above. The advantages of froth test are simple, time-saving, and easy to conduct and interpret. Formation of honeycomb froth with its height exceeds 2 cm and persists after 10 minutes indicating positive result of froth test (Domingo *et al.*, 2009). Refer to Table 4.6(a), the height of honeycomb froth of Shandong peanut shell, Menglembu peanut shell and peanut root is 2.0 cm, 2.3 cm and 2.3 cm. This result fits the theory which proposed by Domingo *et al.* (2009) proves the presence of saponins in sample analyzed. Formation of honeycomb froth is due to combination of polar side chain (glycones) with non-polar sapogenin (aglycones). Vigorous shaking process promotes the interface or chance to induce combination of hydrophilic and hydrophobic side chains of

saponins. Hence, froth test double confirms the result of foam test and improves the accuracy and reliability of experimental results.

Besides, there is a correlation between froth test and total saponins. Based on experimental result, it can be deduced that total saponins content is directly proportional to the height of honeycomb froth. Greater amount of total saponins in a plant sample will yield a greater height of honeycomb froth. For illustration, peanut root has the greatest amount of total saponins and thus it yields the highest honeycomb froth; whilst height of honeycomb froth of Shandong peanut shell is the lowest due to its lowest content of total saponins.

5.3.3 Percent recovery of saponins extract

Percent recovery is an important standard that determine the purity of sample or substance after a purifying process. As mentioned in result, the percent recovery obtained is satisfactory as more than 90% and close to 100%. High percent recovery indicates the purifying process is perfect and freeze-dried saponins extracts have a high purity with its minimal lost of desirable product. However, the lost of these minimal saponins might be due to long and tedious of extraction procedure (Guo, Song, Liu & Liu, 2006). This is because the general extraction procedure includes isolation of saponins from sample, chemical manipulation and followed by spectroscopic analysis, and thus this contributes possible sources for the loss of saponins.

5.3.4 Determination of total saponins

Total saponins is the content of saponins as a whole, such as triterpenoids saponins and steroidal saponins that present in plant materials. Total saponins equivalent to summation of different classes of saponins which present in a sample.

Determination of total saponins in peanut root and shells was conducted by spectrophotometric assay. Vanillin reagent serves as an important mediator in the formation of chromogens. Vanillin in acid mediunm reacts with triterpenoids sapogenin, sterol, bile acids with a hydroxyl group (-OH) at C_3 position and steroidal saponins with or without double bond at C_5 position. This process produces chromogens that can be read at 544 nm. The nature of sugar moieties exerts no influence to formation of chromogen. Regarding different type or variety of sugar moieties in saponins, chromogen that formed from reaction with vanillin still is the same.

According to Table 4.5, total saponins content of Menglembu peanut shell and peanut root is closely approximate to each other, which are 20.00 ± 0.03 mg/ 10 g and 21.20 ± 0.02 mg/ 10 g. Total saponin content of Shandong peanut shell is significantly lower than the other two samples, which is 12.30 ± 0.03 mg/ 10 g. High reliability of result can be proved by coefficients of variation for all samples that below 5%.

As there are no any previous studies in determination of total saponins in peanut shell and root, the obtained experimental result was compared with

reported total saponins content from other type of plant materials. The reported range of total saponins content in root and shell from other plant material is 4.91 – 898 mg/ 10 g (Kao *et al.*, 2008; Dini *et al.*, 2009; Gevrenova, Stancheva, Voynikov, Laurrain-Mattar & Henry, 2010; Man *et al.*, 2010). By making a comparison, the total saponins content obtained in this analysis lie within reported range.

The variation of total saponins content in Shandong peanut shell, Menglembu peanut shell and peanut root matches with theory proposed by Dini *et al.* (2009) regarding the quantitative difference of saponins contents in plant organs. Correspond to distribution of saponins in different plant organs; peanut root has the highest total saponin content among others. This observation can be explained by findings of Felix *et al.* (1990): roots of *Panax ginseng* contains numerous steroidal saponins and triterpenoid saponins which contributes to high level of total saponins content. Total saponins content of Shandong peanut shell and Menglembu peanut shell can be characterized by their bitter taste. The bitter taste of these peanut shells might become feeding deterrent for animal feedstuffs and subsequently affect palability and intake of nutrient (Cheeke, 1989).

Total saponins content as well as total steroidal saponins in peanut root and shell are influenced by several factors, such as plant species, part of plant that examined, genetic origin of plant, physiological age of plant, state, environmental and agronomic factors (Felix *et al.*, 1990). Besides, it is also influenced by post-harvest treatment such as storage and processing techniques.

Dehulling, soaking, germination and fermentation are examples of processing techniques that reduce saponins content in foodstuffs (Redden *et al.*, 2005).

Lastly, total saponins contents obtained from samples are compared with reported toxicity threshold of saponins. In general, level of saponins that below 1 g/kg (or equivalent to 1%) in diet are safe to be consumed and unlikely to exerts anti-nutritional effects to living organisms (Francis *et al.*, 2001). By conversion of total saponins content (μg/ 10 g) to g/g, the percentage of total saponins content of Shandong peanut shell, Menglembu peanut shell and peanut root are 0.001%, 0.002% and 0.021%. All these values are lower than 1% and this shows that saponins content of these samples will not possess hazardous effect to living organisms if used as animal feedstuffs.

5.3.5 Determination of total steroidal saponins

Steroidal saponins is a subclass of saponins accompanying with triterpenoid saponins. Steroidal saponins or "steroids building blocks" consists of steroid structure with a fat-soluble steroidal nucleus (Cheeke, 1989). Total steroidal saponins is a summation of steroidal saponins with different steroidal glycosides, such as furastanol saponins, spirotanol saponins, polipodo saponins and nugatigenin saponins (Hostettman & Marston, 1995).

Total steroidal saponins was determined by spectrophotometric assay due to simplicity and sensitivity. In this assay, anisaldehyde and ethyl acetate in acidic medium are important to react with steroidal saponins. This reaction leads to

formation of yellowish coloured complex that has the maximum absorption spectrum at 430 nm (Baccou *et al.*, 1977).

Table 4.5 depicts it shows total steroidal saponins of Shandong peanut shell, Menglembu peanut shell and peanut root. Peanut root has the highest total steroidal saponins, which is 2.56 ± 0.00 mg/ 10 g with coefficient of variation of 0.07; followed by Menglembu peanut shell that consists of 2.50 ± 0.00 mg/ 10 g. Its coefficient of variation is 0.11. Shandong peanut shell contains the least amount of total steroidal saponins, which is 0.90 ± 0.00 mg/ 10 g with 0.48 as its coefficient of variation.

Comparison was conducted between obtained experimental total steroidal saponins with reported total steroidal saponins from different parts of *Dioscorea pseudojaponica*. The reported range of total steroidal saponins is 0.118 – 3.900 mg/ 10 g (Lin & Yang, 2008). Hence, the experimental total steroidal saponins fall into range of theoretical values. Low value of coefficient of variation below 5% and small value of standard deviation indicates reproducibility and reliability of result.

A good correlation can be established between total steroidal saponins and total saponins content. As mentioned earlier, total steroidal saponins is a small fraction of total saponins content. Due to this, amount of total steroidal saponins influences content of total saponins. In this study, high total steroidal saponins in peanut root yields the highest total saponins content as well.

According to various literature sources, total steroidal saponins is a very special secondary metabolites which is a significantly plant bioactives rather classified as anti-nutritional factors. Total steroidal saponins possess many biological functions, such as anti-carcinogenic, anti-thrombotic, anti-viral, haemolytic, hypocholesterolemic and hypoglycaemic effects (Peng *et al.*, 1996; Zhang *et al.*, 1999; Lin & Yang, 2007). Therefore, high distribution of steroidal saponins in Shandong peanut shell and peanut root is beneficial to industrial production of steroidal drugs. For instance, Diosgenin sapogenin of the yam steroidal saponins was used as raw material for industrial production of steroidal drugs (Djerassi, 1992; Chen & Wu, 1994).

5.4 Alkaloids Content of Peanut Shell and Peanut Root

Determination of alkaloids using thin layer chromatography and gravimetric analysis share the similar extraction scheme. The extraction of alkaloids is based on their basic characters (either by acidic extraction or basic extraction) and solubility patterns (Robert & Wink, 1998). For instance, the salts of most alkaloids are water-soluble; the free bases are insoluble in water and sparingly soluble in organic solvents.

For this study, acidic extraction of alkaloids was carried out. Mild acidic solution was used to extract alkaloids from ground peanut root and shells. Alkaloids compound is converted to salts when react with 10% acetic acid in methanol. The purpose of vacuum evaporation of alkaloids using rotary evaporator to remove methanol as well as to concentrate alkaloids extracts. Upon addition of 30% ammonium hydroxide solution (base), this salt form of

alkaloids will be converted to its basic forms. Subsequently, the ammonium hydroxide precipitates is then washed with 1% ammonium hydroxide solution in which it converts aqueous solution of alkaloids salts to alkaline form and treated with chloroform to remove impurities from alkaloids extracts. Treatment of organic solvent was conducted for three times to ensure maximum purity level can be achieved and to avoid the interference of alkaloids extracts with impurities.

5.4.1 Quantitative evaluation of alkaloids by gravimetric analysis

Gravimetric analysis is a quantitative isolation of a substance by precipitation and weighing of the precipitate. The core of gravimetric analysis formed by: analyte which is the substance to be analysed; precipitating agent which is the medium that precipitate the analyte out from samples. In this case, alkaloids is the analyte while ammonium sulphate solution is the precipitating agent.

To ensure desirable alkaloids precipitated out from samples, ammonium hydroxide solution with very low solubility (K_{sp} very small) added as precipitating agent to facilitate precipitation and precipitate alkaloids out from samples.

Washing of precipitate is one of the important stages in gravimetric analysis. The aim of this step is to remove any impurities that present to contaminate precipitate as complete as possible. In this study, 1% ammonium hydroxide solution serves as washing agent to ensure the complete removal of impurities that might present and interfere with precipitates. Besides, precipitate obtained

is a salt of weak acid as 10% acetic acid in methanol is used to extract alkaloids out from sample. The precipitate which is a salt of weak acid is lightly soluble and has a high tendency to be hydrolysed; the product of hydrolysis is a base. Thus, it is essential to choose 1% ammonium hydroxide solution (base) as washing agent to prevent hydrolysis.

After filtration, filter paper was heated at 60 ℃ for 30 minutes. This is due to two purposes: first, for drying purpose to remove all moisture on filter paper; second, conversion of precipitate to more stable chemical form prior to weighing. After heating session, it is important to transfer filter paper to dessicator instantly just after took out from oven. Some modifications of procedure have been made to minimize possible source of error, in which filter paper was placed into pre-weighed moisture dish for drying and weighing session. This is because to avoid dried filter paper to reabsorb moisture content from atmosphere or other environmental sources and to prevent inaccuracy of result. The aim of repeating drying and weighing purpose is to obtain a constant mass for alkaloids precipitate. The weight of moisture dish was subtracted from combined mass to yield the actual mass of alkaloids precipitate. Total alkaloids can be expressed as a percentage form of mass of alkaloids precipitate.

The percentage of alkaloids which obtained in this analysis give an expression of a summation of total alkaloids that present in Shandong peanut shell, Menglembu peanut shell and peanut root regarding it groups and types. Refer to Table 4.4.2, the percentage of alkaloids of peanut root is the highest, which

is 23.77 ± 0.02 % with coefficient of variation of 0.08. This parallel with determination of alkaloids using thin layer chromatography which will be discussed in later part in which only peanut root was found to consist of alkaloids. However, Shandong peanut shell and Menglembu peanut shell with negative result in qualitative evaluation test was found to give positive result in gravimetric analysis. Both of these samples contain low percentage of alkaloids, which is below 10%. Menglembu peanut shell possesses a higher percentage of alkaloids than Shandong peanut shell, which is 8.35 ± 0.01% with coefficient of variation of 0.12. Shandong peanut shell only contains 4.46 ± 0.01% of alkaloids with 0.22 as its coefficient of variation. Coefficient of variation for all samples that below 5% indicates the accuracy of result and a low level of standard deviation provides a high reliability of result.

The percentage of alkaloids found in peanut root matches with numerous theoretical concepts regarding distribution of alkaloids in plant organs. Combination results of qualitative evaluation test and gravimetric analysis double confirms the presence of alkaloids in peanut root. From gravimetric analysis, it is believed that the high percentage of alkaloids in peanut root might implies that the level of Berberine is extremely high and there are possibilities that other types of alkaloids that beyond determination of thin layer chromatography might present as well. Numerous literature reviews have reported that alkaloids are highly distributed in root system of a plant (Robert & Wink, 1998; Makkar *et al.*, 2007). As mentioned in section 5.4.2, peanut root is extremely important to the fitness and survival of peanut plant. The damage of peanut root system by herbivorous attack, insects attack or any

environmental hazards will give adverse effect to peanut plant (Robert & Wink, 1998). Thus, the high percentage of alkaloids in peanut root through gravimetric analysis is acceptable.

For Shandong peanut shell and Menglembu peanut shell, both samples possess low percentage of alkaloids which lower than 10%. This shows that a low amount of alkaloids present in peanut shells although there is no any result can be obtained in thin layer chromatography. It is suspected that type of alkaloids present in peanut shells might not be well-known or common alkaloids that can be identified by thin layer chromatography. Menglembu peanut shell has a higher percentage of alkaloids in compare to Shandong peanut shell. This might be due to sharper bitter taste of Menglembu peanut shell. Bitter taste is one of the characteristic of alkaloids and the role of bitterness is moderately important in plant defence as well as plant - animal relationship. Bitter taste of peanut shell serves as feeding deterrent in plant foodstuffs (Smith, 1972; Fattorosso & Taglialatela, 2007). Due to this, it is advisable to modify bitter taste of peanut shells through processing techniques in order for it to be served as animal feedstuffs.

Care must be taken into account in conducting gravimetric analysis. An over high of percentage of alkaloids might be due to incomplete precipitation process or precipitation of other undesirable compounds. In contrast, percentage of alkaloids which is too low might be due to incomplete drying process. In this analysis, the percentage of alkaloids which was found in peanut root and shells are in moderate range. As compare to findings reported by

Adeniji, Orjikwe and Ehiagbonare (2008), the percentage of alkaloids of some common foodstuffs is in 11.40 – 29.50 %. For Shandong peanut shell and Menglembu peanut shell, the low percentage of alkaloids in these samples is understandable as peanut shells are less important to plant's fitness than peanut root. Hence, alkaloids which serve as plant defence compound less distributed in peanut shell as compare to peanut root (Robert & Wink, 1998).

5.4.2 Identification of Alkaloids Classes by Thin Layer Chromatography

Referring to Figure 4.6, only fluorescent yellow bands can be observed in peanut root, while there are no bands being observed in Shandong peanut shell and Menglembu peanut shell. The retention factor for fluorescent yellow bands of peanut root is 0.65 ± 0.02. Low value of coefficient of variation indicates the precision of triplicate readings of retention factor. Based on fluorescence behaviour under ultraviolet light and retention factors obtained, it can be concluded that Berberine is the class of alkaloids present in peanut root.

Berberine (Figure 5.1) is a quaternary ammonium salt and categorized as isoquinoline alkaloids. Yellow Berberine is coloured in nature. Berberine is derived from aromatic amino acids such as phenylalanine, tyrosine and tryptophan which has ultraviolet-absorbing property and thus presents as fluorescent yellow under ultraviolet light (Robert & Wink, 1998). Berberine with aromatic rings in their structures absorbs at longer wavelength and has λ_{max} at 228 nm. In term of retention factor, the standard reference value for Berberine is 0.70 (Makkar *et al.*, 2007). The average of retention factor of peanut root is 0.65 ± 0.02 and closely approximate to this value. The

coefficient of variation will be 3.06% which below than 5% and this indicates that the standard deviation obtained is negligible and results obtained is highly precise.

Corresponds to distribution of Berberine in plant organs, Berberine is a class of alkaloids usually found only in roots, rhizomes, stem and bark (Robert & Wink, 1998). This is the reason of Berberine only can be determined in peanut root instead of Shandong peanut shell and Menglembu peanut shell. The obtained experimental result is again in parallel with theory.

Berberine offers a wide range of benefits and applications. Due to its colour property, Berberine can be applied as dye in wool, wood and leather; heparins stain in mast cells. Berberine also used as traditional medicine and diertary supplement, as it exerts anti-inflammatory activity against fungal infections, parasites, viral infections and bacterial infections; lowers blood cholesterol, LDL cholesterol, aterogenic apolipoprotein (apo B) and triglycerides. Berberine can be used as antibiotic as it inhibits growth of bacteria, such as *Staphylococcus aureus* and multidrug resistance pumps. Berberine also applied as eye drop formulations.

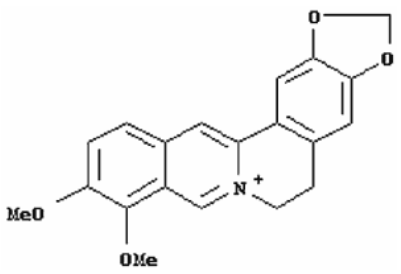


Figure 5.1: Chemical structure of isoquinoline alkaloids –Berberine.

In terms of absence of any alkaloids in Shandong peanut shell and Menglembu peanut shell, it can be explained with several reasons. First and foremost, the alkaloids present in peanut shells might not be the common or well-known alkaloids that can be identified by thin layer chromatography with known retention factor and unusual colour property in ultraviolet region. Thin layer chromatography which used in this analysis is only eligible for identification of common alkaloids, such as cytosine, nicotine, tomatine, morphine, solanine, codeine, berberine, strychnine, thebaine, atropine, quinine and coniine.

Moreover, previous studies reported that over 21000 types of alkaloids have been identified and constitutes the largest natural-occurring compound among the nitrogen-containing secondary metabolites (Fattoroso & Tagialatela, 2007). As supporting by literature sources by Makkar *et al.* (2007), identification or quantification of alkaloids cannot rely on a single method. This is due to abundance and high hetergenousity of alkaloids. In addition, extraction procedure is based on each alkaloid's basic character and solubility (Robert & Wink, 1998). Since there is a wide range of solubility and characteristic of alkaloids, general screening procedure may fail to detect particular class of alkaloids. Therefore, it is possible to have uncommon alkaloids present in peanut shells and cannot be identified by thin layer chromatography. However, due to shortage of sophisticated laboratory instrument and limited project time length, it is difficult to conduct other methods for identification of alkaloids in these samples.

Secondly, there might be some common alkaloids present but not prominent in peanut shell, for instance, Berberine is predominant in peanut root, but not found in peanut shell. Different alkaloids distribute in different plant organs. It can be supported by literature report by Robert and Wink (1998) in which alkaloids were found to be present in plant organs that susceptible for herbivorous attack and the damage to organs is significant to plant's fitness, such as young growing tips, peripheral cell layers of stems and roots. In comparison to peanut root which acts as important medium in absorption and transportation of water to whole plant, it is assumed that there might be absent of common alkaloids in peanut shell, as the damage of single peanut shell does not exerts an adverse effect to plant's fitness.

In conclusion, Berberine was found in peanut root but there are no any alkaloids can be determined in Shandong peanut shell and Menglembu peanut shell. Since this is qualitative evaluation test, the adverse effect of samples towards living organisms if utilized as animal feedstuffs cannot be determined. Theoretical sources reviewed that the Exposure Dose, ED_{50} of Berberine is 0.25% - 0.50%. For future study, spots appear on chromatography plate can be taken for further analysis by densitometry to obtain quantitative result.

5.5 Future Study

Several investigations can be extended from this current analysis to ensure the suitability of peanut shells and root to use as animal feedstuffs. Further analysis can be conducted to reduce anti-nutritional factors in peanut shells and root. Several methods such as cooking, fermentation, germination, soaking and

dehulling can be carried out and study the effect of each methods to anti-nutritional factors that present in peanut shells and root. This is important for designation of processing technique in manufacture animal feedstuffs from peanut shell and peanut root.

For saponins and alkaloids assay, individual class of saponins and alkaloids can be further analysed by more sophisticated techniques such as HPLC and ESI-MS/MS. Besides, the structure of each individual class of saponins and alkaloids can be investigated by using NMR to obtain structure-functional relationship. However, several problems should be taken into consideration before utilization of peanut by-products as animal feedstuffs, such as bitter taste of saponins and alkaloids that acts as feeding deterrent.

Lastly, to achieve the aim of utilization of peanut by-products as animal feedstuffs, further investigation can be conducted by include animal model in feeding experiments. The anti-nutritional effects can be studied by varies different doses intake of phytic acid, saponins and alkaloids to animals. Subsequently, toxicity threshold of each anti-nutritional factor can be determined based on feeding experiment.

CHAPTER 6

CONCLUSIONS

In brief, the objective of this analysis has been achieved in which anti-nutritional factors such as phytic acid, saponins and alkaloids were found in peanut shells and root. The moisture content for Shandong peanut shell, Menglembu peanut shell and peanut root are 9.56% - 9.94%.

Phytic acid level in peanut root and shells are 0.107 – 0.165 g/ 100g. The amount of phytic acid in peanut by-products fits assumptions in which its value is lower than reported range of phytic acid level in peanut seeds. Phytic acid content in Shandong peanut shell, Menglembu peanut shell and peanut root are not able to exert anti-nutritional effects to living organisms as it is lower than theoretical toxicity threshold, which is 1%.

For saponins assay, positive result of foam test and froth tests determines the presence of saponins qualitatively in peanut by-products. Total steroidal saponins for all samples are 0.90 – 2.57 mg/ 10g; total saponins content lies in the range of 12.3 – 21.2 mg/ 10g. Both results are accurate as matches with theoretical range of plant materials. Total steroids saponin and total saponins content of peanut by-products unlikely to poses risks in living organism as it below theoretical threshold, which is 1 g/kg.

In alkaloids assay, the presence of alkaloids in samples is determined by thin layer chromatography. Only Berberine can be found in peanut root. Berberine

is an isoquinoline alkaloid that commonly distributed in peanut root instead of peanut shell. There are no any class of alkaloids can be identified in Shandong peanut shell and Menglembu peanut shell.

Total alkaloids content for all samples are 4 – 24%, in which peanut root has the highest total alkaloids content. Shandong peanut shell and Menglembu peanut shell shows positive result in gravimetric analysis probably due to presence of uncommon alkaloids that beyond identification scope of thin layer chromatography. Toxicity level of alkaloids in living organism cannot be identified as gravimetric analysis only provide total alkaloids content; whereas toxicity threshold of each alkaloids class only available in literature sources.

In conclusion, phytic acid, saponins and alkaloids can be determined in peanut by-products. Concentration of phytic acid and saponins unlikely exerts anti-nutritional effects to living organism; toxicity level of alkaloids can be investigated in future studies. This analysis provides a foundation base for future investigation to utilize peanut by-products as animal feedstuffs.

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APPENDIX A

Experimental raw data of Shandong peanut shell in moisture content assay.

Replicates / Parameters	Shandong Peanut Shell		
	First	Second	Third
Initial Sample Weight (g)	5.0000	5.0000	5.0000
Dry Sample Weight after 5 hours (g)	4.5221	4.5223	4.5219
Dry Sample Weight after 6 hours (g)	4.5221	4.5223	4.5219
Moisture Content (%)	9.5580	9.5540	9.5620
Average of Moisture Content (%)		9.5580	
Standard Deviation		0.0040	
Coefficient of Variation (%)		0.0418	

Experimental raw data of Menglembu peanut shell in moisture content assay.

Replicates / Parameters	Menglembu Peanut Shell		
	First	Second	Third
Initial Sample Weight (g)	5.0000	5.0000	5.0000
Dry Sample Weight after 5 hours (g)	4.5253	4.5249	4.5248
Dry Sample Weight after 6 hours (g)	4.5253	4.5209	4.5208
Moisture Content (%)	9.4940	9.5020	9.5020
Average of Moisture Content (%)		9.5000	
Standard Deviation		0.0046	
Coefficient of Variation (%)		0.0486%	

Experimental raw data of Menglembu peanut shell in moisture content assay.

Replicates / Parameters	Peanut Root		
	First	Second	Third
Initial Sample Weight (g)	5.0000	5.0000	5.0000
Dry Sample Weight after 5 hours (g)	4.5031	4.5028	4.5033
Dry Sample Weight after 6 hours (g)	4.5031	4.5028	4.5033
Moisture Content (%)	9.9380	9.9440	9.9340
Average of Moisture Content (%)		9.9390	
Standard Deviation		0.0050	
Coefficient of Variation (%)		0.0506	

APPENDIX B

Absorbance values for calibration curve of iron (III) nitrate nonanhydrate.

Vol. of Stock (mL)	Num. of mol of Iron	Amount of Iron(g)	Absorbance
2.5	1.8×10^{-6}	1.005×10^{-4}	0.067
5.0	3.6×10^{-6}	2.010×10^{-4}	0.156
7.5	5.4×10^{-6}	3.015×10^{-4}	0.286
10.0	7.2×10^{-6}	4.020×10^{-4}	0.391
12.5	9.0×10^{-6}	5.026×10^{-4}	0.535

Absorbance values of Shandong peanut shell, Menglembu peanut shell and peanut root at 480 nm.

Samples \ Absorbance	Absorbance Value for Replicates			Average of Absorbance Value
	1	2	3	
Shandong Peanut Shell	0.110	0.109	0.109	0.109
Menglembu Peanut Shell	0.071	0.072	0.071	0.071
Peanut Root	0.107	0.107	0.106	0.107

APPENDIX C

Height of honeycomb froth for different samples in froth test.

Sample	Height of Honeycomb Froth			Average
	1	2	3	
Shandong Peanut Shell	2.0	1.9	2.0	2.0
Menglembu Peanut Shell	2.3	2.2	2.3	2.3
Peanut Root	2.3	2.3	2.3	2.3

Absorbance values of calibration curve of total saponins.

Volume of Diosgenin (µL)	Amount of Diosgenin (µg)	Absorbance at 544 nm
0	0	0.000
100	50	0.161
125	62.5	0.192
150	75	0.219
175	87.5	0.298
200	100	0.322
225	112.5	0.400
250	125	0.492

Absorbance values of Shandong peanut shell, Menglembu peanut shell and peanut root at 544 nm.

Absorbance / Samples	Absorbance Value for Replicates			Average of Absorbance Value
	1	2	3	
Shandong Peanut Shell	0.430	0.428	0.430	0.429
Menglembu Peanut Shell	0.702	0.700	0.701	0.701
Peanut Root	0.743	0.742	0.743	0.743

Absorbance values of calibration curve of total steroidal saponins.

Volume of Diosgenin (µL)	Amount of Diosgenin (µg)	Absorbance at 430 nm
0	0	0.000
20	2	0.033
40	4	0.122
60	6	0.251
80	8	0.297
100	10	0.328

Absorbance values of Shandong peanut shell, Menglembu peanut shell and peanut root at 430 nm.

Absorbance / Samples	Absorbance Value for Replicates			Average of Absorbance Value
	1	2	3	
Shandong Peanut Shell	0.316	0.314	0.317	0.316
Menglembu Peanut Shell	0.879	0.880	0.878	0.879
Peanut Root	0.900	0.901	0.900	0.900

APPENDIX D

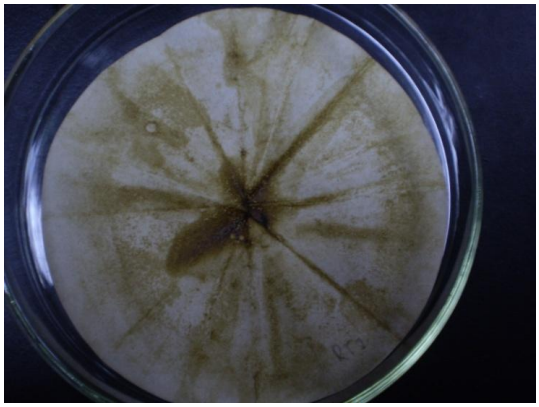
R_f values and colour properties of some well-known alkaloids.

Alkaloids	R_f on paper	R_f on TLC plate	Behaviour in UV light	Recommended reagent for detection	Spectral max (nm) in 0.1 M H_2SO_4
Cytisine	03	32	Blue	Dragendoff	303
Nicotine	07	57	Absorbs	Iodoplatinate	260
Tomatine	08	62	Invisible	Iodoplatinate	-
Morphine	14	34	Absorbs	Iodoplatinate	284
Solanine	15	52	Invisible	Marquis	-
Codeine	16	35	Absorbs	Iodoplatinate	284
Berberine	25	07	Fluorescent Yellow	Iodoplatinate	228
Strychine	30	22	Absorbs	Iodoplatinate	254
Thebaine	32	41	Absorbs	Iodoplatinate	284
Atropine	37	18	Absorbs	Iodoplatinate	258
Quinine	46	52	Bright blue	Iodoplatinate	250
Coniine	56	26	Invisible	Iodoplatinate	268

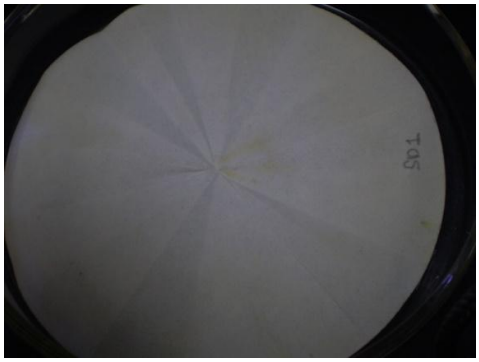
Experiment raw data of Shandong peanut shell, Menglembu peanut shell and peanut root for gravimetric analysis.

Parameter / Sample	Replicate	Weight of filter paper before Drying (g)	Weight of Filter paper after Drying (g)	Weight Differences before & after drying (g)	Percentage of Alkaloids Precipitate (%)
Shandong Peanut Shell	First	0.5429	0.5874	0.0445	4.45
	Second	0.5257	0.5704	0.0447	4.47
	Third	0.5339	0.5785	0.0446	4.46
Menglembu Peanut Shell	First	0.5330	0.6166	0.0836	8.36
	Second	0.5373	0.6207	0.0834	8.34
	Third	0.5356	0.6190	0.0834	8.34
Peanut Root	First	0.5490	0.7867	0.2377	23.77
	Second	0.5280	0.7655	0.2375	23.75
	Third	0.5494	0.7872	0.2378	23.78

APPENDIX E



Dried filter paper with alkaloids precipitate from peanut root.



Dried filter paper with alkaloids precipitate from Shandong peanut shell.

Dried filter paper with alkaloids precipitate from Menglembu peanut shell.