THE EFFECTS OF GAMMA IRRADIATION ON MICROBIOLOGICAL SAFETY, BIOLOGICAL ACTIVITIES AND GALLIC ACID CONTENT OF *EUODIA MALAYANA*, *GNETUM GNEMON* AND *KHAYA SENEGALENSIS*

MUHAMMAD HIDHIR BIN KHAWORY

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by

MUHAMMAD HIDHIR BIN KHAWORY

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-	minus or negative or to
+	plus
x	times
/	divide or per
%	percentge
&	and
^{o}C	degree Celcius
*	asterisk
α	alpha
β	beta
γ	gamma
λ	wavelength
$\bar{\upsilon}$	wavenumber
υ	frequencies
>	greater than
±	plus-minus
=	equal
<	less than
≤	less than and equal to
®	registered
™	trade mark
µm	micrometer
µl	microliter
µg/ml	microgram per milliliter
ACN	acetonitrile
AJS	Agilent jet stream
ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
API-MS	Atmospheric Pressure Ionization Mass Spectrometry
ATCC	American Type Culture Collection
C	Coulomb

C8	carbon eight
C18	carbon eighteen
cells/ml	cells per milliliter
CFU	colony forming unit
CFU/ml	colony forming unit per milliliter
CL	cathodoluminescence
cm^{-1}	centi per meter
cm^2	square centimeter
CO_2	carbon dioxide
DAD	diode array detector
DMSO	dimethyl sulfoxide
DOH	Departement of Health
DPPH	diphenylpicrylhydrazyl
EC_{50}	half maximal effective concentration
EMEA	European Medicines Agency
EMEM	eagle's minimal essential medium
ESI	electrospray ionization
EtOH	ethanol
FAO	Food and Agriculture Organization
FBS	fetal bovine serum
FID	flame ionization detector
FTIR	Fourier Transform Infrared
g	gram
GA	gallic acid
GAE	gallic acid equivalent
g/cm^3	gram per cubic centimeter
g/mol	gram per mole
Gy	Gray
H_2O	water
HACCP	hazard analysis critical control points
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IAEA	International Atomic Energy Agency

IC_{50}	half maximal inhibitory concentration
id	internal diameter
i.e.	example
IPharm	Malaysian Institute of Pharmaceutical and Nutraceutical
IPNAT	IPharm Nature
IR	Infrared
ISO	International Organization for Standardization
J/kg	Joule per kilogram
kCi	kilocurie
kGy	kiloGray
kGy/hr	kiloGray per hour
kPa	kilopascal
krad	kilorad
L	Liter
LC	liquid chromatography
LCMS	Liquid Chromatography Mass Spectrometry
M	Molarity
MEM	minimal essential medium
MeOH	methanol
mg	milligram
mg GAE/g	milligram gallic acid equivalent per gram
mg/ml	milligram per milliliter
ml	milliliter
ml/min	milliliter per minute
MLT	microbial limit test
mm	millimeter
mM	millimolar
Mrad	Millionrad
m/v	mass per volume
m/z	mass to charge ratio
N/A	not available
nm	nanometer
OD	optical density

PBS	phosphate buffer saline
PCs	phenolic compounds
pH	potenz hydrogen
ppm	part per million
psi	pounds per square inch
Q-TOF	quadrupole-time of flight
R	Roentgen
R^2	coefficient of a linear regression
RFU	relative fluorescence unit
RH	relative humidity
RID	refractive index detector
RM	Ringgit Malaysia
rpm	rotation per minute
RSD	relative standard deviation
SDS	sodium dodecyl sulfate
sp.	species (singular)
spp.	species (plural)
TLC	thin layer chromatography
TPC	total phenolic content
UK	United Kingdom
U/ml	units per milliliter
USA	United States of America
USD	United States Dollar
UV	ultraviolet
v/v	volume per volume
WHO	World Health Organization

LIST OF APPENDICES

KESAN-KESAN PENYINARAN GAMA TERHADAP KESELAMATAN MIKROBIOLOGI, AKTIVITI-AKTIVITI BIOLOGI DAN KANDUNGAN ASID GALIK BAGI *EUODIA MALAYANA*, *GNETUM GNEMON* DAN *KHAYA SENEGALENSIS*

ABSTRAK

Tumbuh-tumbuhan ubatan seperti herba dan rempah sering dicemari dengan paras bakteria, kulat dan yis yang tinggi. Oleh yang demikian, tumbuh-tumbuhan ubatan boleh dikontaminasi dengan menggunakan penyinaran gama bagi mensterilkan mikroorganisma-mikroorganisma secara berkesan. Matlamat kajian ini adalah untuk menganalisis kesan-kesan bagi rawatan radiasi gama terhadap tiga jenis tumbuh-tumbuhan ubatan iaitu *Euodia malayana*, *Gnetum gnemon* dan *Khaya senegalensis* pada dua bentuk yang berbeza; ekstrak daun metanol dan dedaun kering. Kajian ujian had mikrob menunjukkan dos minimum dan maksimum sinaran gama yang sesuai bagi setiap ekstrak tumbuhan adalah 6-12 kGy manakala bagi setiap dedaun kering adalah 9-13 kGy. Kajian kestabilan menunjukkan bahawa tidak ada pencemaran mikrob selepas disimpan selama satu tahun di bawah suhu dan kelembapan yang terkawal selepas rawatan radiasi gama. Bagi analisis komponen kimia, analisis kualitatif dengan menggunakan Kromatografi Cecair Prestasi Tinggi (KCPT) mendedahkan kehadiran sebatian asid galik pada minit ke 2.173 bagi setiap tumbuhan. Analisis kuantitatif pula menunjukkan bahawa peratusan kepekatan asid galik dalam setiap tumbuhan meningkat dengan ketara daripada 0.024%-0.053% sebelum dirawat kepada 0.03%-0.064% selepas dirawat dengan sinaran gama. Keputusan ini telah disahkan oleh Jumlah Kandungan Fenolik (JKF) analisis berdasarkan pengukuran kalorimetri yang ditentukan dengan menggunakan reagen Folin-Ciocalteu di mana asid galik dijadikan sebagai rujukan. Asai DPPH (2,2-

difenil-1-pikril-hidrazil) telah dijalankan dengan menggunakan vitamin C dan asid galik sebagai kawalan positif untuk menentukan sifat antioksidan bagi setiap tumbuhan. Nilai-nilai kepekatan berkesan (EC_{50}) bagi tumbuhan *Khaya senegalensis* berkurang dengan ketara ($P \leq 0.005$) daripada 44.510 µg/ml sebelum dirawat kepada 24.691 µg/ml selepas dirawat dengan sinaran gama di mana ia menunjukkan peningkatan aktiviti memerangkap radikal bebas. Asai sitotoksisiti secara *in vitro* dengan menggunakan sel fibroblas (L929) telah dijalankan ke atas setiap tumbuhan untuk menentukan kesan ketoksikan. Nilai-nilai kepekatan bagi perencat (IC_{50}) bagi setiap tumbuhan adalah > 90 µg/ml di mana ia menunjukkan sebagai tak bertoksik. Ini menunjukkan tiada kesan yang ketara bagi sebelum dan selepas dirawat dengan penyinaran gama di mana natrium dodesil sulfat bertindak sebagai kawalan positif di dalam kajian ini. Hasil daripada kajian ini menunjukkan bahawa penyinaran gama tidak mempunyai kesan buruk malah meningkatkan keselamatan mikrobiologi dan memelihara aktiviti sitotoksik yang rendah di samping meningkatkan aktiviti antimikrob, aktiviti antioksidan dan jumlah kandungan fenolik bagi *Euodia malayana*, *Gnetum gnemon* dan *Khaya senegalensis*. Oleh itu, proses penyinaran gama adalah selamat, bersih, tidak aktif secara kimia dan berkesan digunakan untuk pensterilan bagi tumbuh-tumbuhan ubatan seperti herba dan rempah.

THE EFFECTS OF GAMMA IRRADIATION ON MICROBIOLOGICAL SAFETY, BIOLOGICAL ACTIVITIES AND GALLIC ACID CONTENT OF *EUODIA MALAYANA*, *GNETUM GNEMON* AND *KHAYA SENEGALENSIS*

ABSTRACT

Medicinal plants such as herbs and spices are often contaminated with high levels of bacteria, fungus and yeasts. Therefore, medicinal plants can be decontaminated using gamma irradiation to sterilize the microorganisms effectively. The aim of this study is to evaluate the effects of gamma radiation treatment on three medicinal plants, namely *Euodia malayana*, *Gnetum gnemon* and *Khaya senegalensis* at two different forms; methanol leaf extracts and dried leaves respectively. The microbial limit test studies indicated the suitable dosage of minimum and maximum gamma irradiation was 6-12 kGy for leaf extracts and 9-13 kGy for dried leaves of all the medicinal plants. The stability studies demonstrated that there was no microbial contamination one year after gamma radiation treatment when the plants were stored under controlled temperature and humidity. For the chemical constituent analysis, qualitative analysis using High Performance Liquid Chromatography (HPLC) revealed the presence of gallic acid compound at retention time of 2.173 minutes in each plant. Quantitative analysis showed that the percentage of gallic acid concentration in each plant increased significantly from 0.024%-0.053% before treatment to 0.03%-0.064% after treatment with gamma radiation. This result was confirmed by total phenolic content (TPC) analysis based on calorimetric measurements determined using the Folin-Ciocalteu reagent with gallic acid used as the reference. DPPH (2,2-diphenyl-1-picryl-hydrazyl) assay was conducted by using vitamin C and gallic acid as the positive controls to determine the antioxidant property of each plant. Effective concentration (EC_{50}) values of

Khaya senegalensis plant reduced significantly ($P \leq 0.005$) from 44.510 µg/ml before treatment to 24.691 µg/ml after treatment with gamma radiation, which indicate an increase of free radical scavenging activity. *In* vitro cytotoxicity assay by using fibroblast (L929) cell lines was performed on each plant to determine the toxicity effect. The concentration of inhibitor (IC_{50}) values of each plant is > 90 µg/ml which indicates as nontoxic. This showed no significant effect before and after treatment with gamma irradiation where sodium dodecyl sulfate (SDS) acts as positive control in this study. From this study, it shows that gamma irradiation had no adverse effect thus improved the microbiological safety and sustaining the low cytotoxicity activity as well as enhancing the antimicrobial activities, antioxidant activity and total phenolic content of *Euodia malayana*, *Gnetum gnemon* and *Khaya senegalensis*. Hence, gamma irradiation process is safe, clean, chemically inert and effective to be used for sterilization of medicinal plants such as herbs and spices.

CHAPTER 1

INTRODUCTION

Plant has been used for ages for food, shelter, treat disease and human disorders. Malaysia is well-known as one of the largest biodiversity that has many species of plant with medicinal properties. Medicinal plant could be defined as plants which may have medicinal properties and many of them were collected from forest (Batugal *et al*., 2004). Medicinal plants are one of the valuable non-timbers in the forest. Medicinal plants have been the mainstay of traditional herbal medicine amongst rural dwellers worldwide since antiquity to date. Traditional medicine is an important part in Malaysian culture and were practiced by ancestors long before the introduction of modern medicine (Abdul Aziz *et al*., 2003) .

Medicinal plants are currently in considerable significance view due to their special attributes as a large source of therapeutic phytochemicals that may lead to the development of novel drugs (Sulaiman *et al*., 2013). Most of the phytochemicals from plant sources such as phenolics and flavonoids have been reported to have positive impact on health and cancer prevention. Phytochemicals plant-based substances and secondary metabolites such as phenolic compounds that are ubiquitous in the plant kingdom can be beneficial to human health, and some have been associated with reduced risk of major chronic diseases (Liu, 2004).

The world demand for natural products using plants as medicine, cosmetics and food supplements has been increasing rapidly. However, a serious problem with plants is microbial contamination with pathogenic bacteria which can result in serious food-borne illnesses (Piyanuch and Jarunee, 2012). Therefore,

decontamination of plant materials is important to increase the safety of medicinal plants. Medicinal plants can be decontaminated using irradiation such as gamma rays (Fang and Wu, 1998). Gamma rays have been used as an effective way to maintain the quality of food for a long time. In addition, radiation treatments of food can kill and sterile insects as well as they can prevent reproduction of food-born parasites (Moy and Wong, 1996).

1.1 Problem Statement

Throughout history, medicinal plants make an important contribution to mankind health. According to the World Health Organization (WHO), about 65 to 80% of the world's population living in developing countries still depends essentially on medicinal plants (herbs) for primary healthcare. Even in recent years, in the developing or developed countries, the demand for plant natural products as ingredients in cosmetics, food supplements and medicines have increased rapidly (Piyanuch and Jarunee, 2012).

Malaysia is blessed with one of the most diverse and oldest flora in the world. Its flora is a rich source of medicinal plants with bioactive molecules possessing interesting pharmacological activities. Unfortunately, medicinal plants such as herbs and spices are often contaminated with high levels of bacteria, fungus, moulds and yeasts (Andrzej and Wojciech, 2005). These pathogenic microorganisms may grow on some herbs and spice plants as well as on the surface and inside of the plant's tissue. If untreated, both herbs and spices will cause a rapid spoilage of the products that affect health and have economic impacts (Piyanuch and Jarunee, 2012). In addition, these infected specimens will give false and misleading results in research (Atanasov *et al*., 2015).

Medicinal plants can be decontaminated using gamma irradiation and it has been one of the most popular methods used in commercial preparations for medicinal plants due to its efficiency (Fang and Wu, 1998). Gamma rays are a type of electromagnetic radiation which originates within the nucleus of a radioisotope (Piyanuch and Jarunee, 2012). The gamma process can sterilize effectively in order to kill microorganisms on a wide variety of products.

Gamma irradiation has been comprehensively studied to reduce the microbial contamination of herbs and spices. The use of ionizing radiation is to inactivate food-borne microorganisms that can reduce quality losses during storage as well as to guarantee the hygienic quality of several foodstuffs such as herbs, meat, poultry and spices (Moy and Wong, 1996). Such, decontamination by ionizing radiation may result in safer, cleaner and better quality of herbs and spices (Andrzej and Wojciech, 2005).

1.2 Goal of the Study

Gamma irradiation can be useful for the alteration of physiological characters as reviewed by Kiong Ling *et al*. (2008). Gamma irradiation also can interact with atoms and molecules to create free radicals in cells that are able to modify important components in the plant cells (Kovács & Keresztes, 2002). These radicals have been demonstrated to affect the anatomy, biochemistry, morphology and physiology of plants, depending on the irradiation dosage (Ashraf *et al*., 2003). The effects consist of an alteration in photosynthesis, modulation of the antioxidative system, changes in the plant cellular structure and metabolism as well as changes in malondialdehyde (MDA) levels as marker of free radicals and enhancement of phenolic compounds (Kovács & Keresztes 2002; Kim *et al*., 2004; Wi *et al*., 2007; Ashraf, 2009).

A survey of literature revealed that the effects of gamma irradiation on medicinal plants have not been investigated for *Euodia malayana*, *Gnetum gnemon* and *Khaya senegalensis*. In addition, these selected plants are listed in 500 medicinal plants of MyNature 50000 as well as reported to have phenolic compound respectively. Hence, in this project, the aim is to investigate the effect of gamma irradiation on those minuted dry plants. The irradiated plant extracts were also subjected to content, physico-chemical and microbial activities profiling. It is hoped that this study might provide insight into the radiation dosage and activity needed for the prevention of contamination. Therefore, in this project, the specific objectives are:

- To determine the suitable dosage of gamma radiation treatment on the selected plants and their respective extracts in managing contamination of microbial (bacteria, fungus, spores) upon storage.

- To study the microbial profile of minuted dry plants and their respective extracts before and after gamma radiation treatments.

- To study the effect of gamma irradiation on chemical constituents profile of minuted dry plants and their respective extracts.

- To study the biological effect of gamma irradiation on antimicrobial, cytotoxicity and antioxidant activities of chemical constituents profile.

CHAPTER 2

LITERATURE REVIEW

2.0 Herbal Medicine - General Overview

Herbs have been used throughout human history as sources of beauty enhancers**,** fragrances, food as well as medicines. Abdul Aziz *et al*. (2003) stated that the use of herbs as medicine has a long history starting in the Greek civilization in the West to the Arabic, Chinese, and Indian civilizations in the East. Furthermore, the current estimates for herbal related market ranges between USD 40 to 100 billion with an average annual growth rate of 15 to 20%. This includes the use of herbs as cosmetic ingredients, food or food additives and herbal medicines (Gruenwald and Hezberg, 2002).

It is further reviewed that about 25% of drugs prescribed worldwide come from different species of plants where 121 of active compounds have been used predominantly. Besides, there is a growing trend of people moving from synthetic allopathic drugs to herbal cures due to a preference for a wellness oriented self-administered healthcare. In addition, the high pace of life which induces higher stress and reduced free time as well as the prevalence of chronic illnesses that cannot be cured by conventional drugs contributed to the increased use of herbal cures (Ekor, 2014).

In several countries, traditional healing methods have been incorporated into the modern health system. For instance, over 80% of doctors in Germany prescribe a combination of modern medicines with herbs while in Japan, doctors prescribing phytomedicines are allowed to claim under the national health insurance (Joos *et al*., 2012; Katayama *et al*., 2013)**.**

2.1 Herbal Medicine - Malaysia Scenario

Malaysia is well known to have a rich biodiversity and listed as the 12th most biodiverse nation in the world and 4th in Asia with over 15,000 flowering plants and over 3000 species of medicinal plants. Up to now, only about 500 of the 3000 listed medicinal plants are used and even less are being researched scientifically for their medicinal properties and many more have yet to be discovered in-depth through ethno-botanical research (Abdul Aziz *et al*., 2003). The Malaysian market for herbal and natural products has been estimated to be worth RM 4.55 billion in which 90% of the raw material used was imported (Mohamad Setefarzi, 2001).

Furthermore, there is an increasing popularity and demand of local herbs within and outside Malaysia over the years. The market in Malaysia for herbs and plant-based medicine is estimated to grow at a rate of 15% per annum, with the market value rising from RM 7 billion in 2010, RM 15 billion in 2014 and this figure is expected to increase up to RM 29 billion in 2020 (Khamis, 2015). Suffice to note that herbal industry is a growing industry in Malaysia and global markets.

It is a known fact that the Malaysian government has been strongly supporting the development of a Malaysian herbal industry. In the year 2000, the Ministry of Health has released a Traditional or Complimentary Medicine (TCM) Policy with the emphasis of 'rational use', the philosophy in which traditional medicine along with modern medicine can be used simultaneously. As a result, the key driving forces in Malaysian domestic markets are also influenced by the changes in lifestyle, the growing on health and the growing cost of synthetic medicines.

2.2 Medicinal Plants in Malaysia

Medicinal plants offer alternative remedies with tremendous opportunities that provide access and affordable medicine to poor people. Apart from that, the use in herbal industry also increase employment opportunity, generates income as well as encourages foreign exchange for developing countries (Batugal *et al*., 2004). Studies have shown that many traditional healing herbs and plant parts have medicinal values that can be used to alleviate, cure or prevent several human diseases especially in the rural areas.

Malaysia is one of Asian country that is rich in plant genetic diversity where many of these plants used for medicinal purposes. The various races in Malaysia which include Malay, Chinese, Indian as well as indigenous people (various tribes of 'Orang Asli') use these medicinal plants as the basis of health care especially for rural populations. The research on medicinal plants in Malaysia has been existed since the past few decades. Burkhill, as early as 1935 reported about 2000 species out of the 12000 species of vascular plants to have medicinal properties.

There are many species of medicinal plants used in folk remedies by various indigenous people in Malaysia such as ginger (*Zingiber officinale*), turmeric (*Curcuma domestica*), betel leaf (*Piper betel*), pandan leaf (*Pandanus odorus*), asam gelugor (*Garnicia atroviridis*), mengkudu (*Morinda citrifolia*) and pegaga (*Centella asiatica*) in which research have shown that these herbs showed high antioxidant activity (Jayamalar and Suhaila, 1998; Mohd Zin *et al*., 2002; Zainol *et al*., 2003; Chew *et al*., 2009; Thoo *et al*., 2010). According to Mustaffa *et al*. (2011), turmeric (*Curcuma longa*), mistletoe fig (*Ficus deltoidea*), longevity spinach (*Gynura procumbens*) and noni (*Morinda citrifolia*) have an antidiabetic activity for the

treatment of diabetes mellitus. Meanwhile, tamarind (*Tamarindus indica*) exhibits antiobesity effects by lowering the degree of hepatic steatosis in the obesity-induced rats (Azman *et al*., 2012).

Among the Malaysian plants, *Euodia malayana*, *Gnetum gnemon*, *Khaya senegalensis* despite being widely used as edible plants, the scientific research on them are not so extensive compared to other plants. Thus, the current study provides opportunity to delve further on the chemical, microbial and the stability profiles of these plants as well as the effects that gamma irradiation might have on these profiles.

2.2.1 *Euodia malayana*

Euodia malayana as depicted in Figure 2.1 has several local names such as tenggek burung, cabang tiga or tapak itik. It can be found in the temperate and tropical regions of East Asia especially Indonesia and Malaysia (Rasadah and Zakaria, 1988). The plant characteristics are branches and lush with small size of 5 to 7 metres. The leaves are in trifoliate shape with the size of 6 to 15 centimetres length and 3 to 8 centimetres width. It has slightly bitter taste, crunchy young leaves, lemon-lime aroma and pungent. The leaves have been widely used by local people to treat high blood pressure and for general freshness (Othman *et al*., 2014). It is taken by the Malays as ‘ulam’ (popular traditional fresh herbs taken as salad) (Zaifuddin *et al*., 2014). The taxonomy of *Euodia malayana* is shown in Table 2.1.

Figure 2.1: Plant of *Euodia malayana*

Table 2.1: Taxonomy of *Euodia malayana*

Kingdom	**Plantae**
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Sapindales
Family	Rutaceae
Genus	Euodia
Species	Malayana
Scientific Name	*Euodia malayana*

2.2.2 *Gnetum gnemon*

The local name of *Gnetum gnemon* (Figure 2.2) is melinjau. In Indonesia, this plant is called melinjo and their leaves, fruits and seeds are frequently consumed as part of Indonesian cuisine. It is a small to medium size tree which growing from 15 up to 20 metres tall. The leaves are evergreen with 8 to 20 centimetres length and 3 to 10 centimetres width. Meanwhile, the fruit for female strobilus consists of skin and a large nut like seed with 2 to 4 centimetres length inside as opposed to male strobili which are small and arranged in long stalks. The extract of melinjo seeds were reported by Kato *et al*. (2009) to possess free-radical scavenging activity as well as antimicrobial effects against food-contaminating microorganisms and enterobacteria. Japanese scientists found that this plant is not the cause of gout (Espinoza *et al*., 2015). The taxonomy of *Gnetum gnemon* is shown in Table 2.2.

Figure 2.2: Plant of *Gnetum gnemon*

Table 2.2: Taxonomy of *Gnetum gnemon*

Kingdom	**Plantae**
Phylum	Tracheophyta
Class	Gnetopsida
Order	Gnetales
Family	Gnetaceae
Genus	Gnetum
Species	Gnemon
Scientific Name	*Gnetum gnemon*

2.2.3 *Khaya senegalensis*

Khaya senegalensis (Figure 2.3) is commonly known as African mahogany. This plant is native to Africa and Madagascar. The height of the plant is 10 to 15 metres with 2 to 3 metres in diameter. The leaves are alternate with 13 to 33 centimetres length and 3 to 7 centimetres width. The flowers are white with sweet-scented while the fruits are almost spherical and woody capsule with 4 to 6 centimetres in diameter. In Senegal, this plant is used to treat fever and fatigue (Sanogo, 1999). Atawodi *et al*. (2002) reported that the decoction of the bark is extensively used as a febrifuge and antimalarial. In northern Nigeria, the decoction of the stem bark is also used for treatment of stomach disorders, urinogenital diseases, worm infestation and in the treatment of trypanosomiasis (Omar *et al*., 2003). Moreover, it is reported to be an effective agent as an antimicrobial, antiprotozoal agents (Abreu *et al*., 1999), antisickling (Fall *et al*., 1999) as well as gastrointestinal nematocide (Ademola *et al*., 2004). The taxonomy of *Khaya senegalensis* is shown in Table 2.3.

Figure 2.3: Plant of *Khaya senegalensis*

Table 2.3: Taxonomy of *Khaya senegalensis*

Kingdom	**Plantae**
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Sapindales
Family	Meliaceae
Genus	Khaya
Species	Senegalensis
Scientific Name	*Khaya senegalensis*

2.3 Phenolic Compounds

Phenolic compounds (PCs) are abundant in both edible and inedible plants (Wojdyło *et al*., 2007). PCs are categorized as secondary metabolites either resist the pathogenic aggression or shield the plants against UV radiation (Seal, 2016). Sulaiman *et al*. (2013) reported that PCs are the largest group of phytochemicals that account for most of the activity either in plants or plant products. PCs possess a wide range of biochemical activities such as antioxidant, anti-carcinogenic and anti-mutagenic (Marinova *et al*., 2005).

PCs are both phytochemicals plant-based substances and secondary metabolites that a ubiquitous in the plant kingdom (Liu, 2004). Nicholson and Hammerschmidt (1992) indicated that PCs in vascular plants have been considered to be related to active and passive plant defence responses. In addition, PCs are served as plant defence mechanisms that protect plants against microbial infections (Rahman *et al*., 2014). Moreover, Maddox *et al*. (2010) reported that PCs are synthesized in response to ecological and physiological pressures which include pathogen and insect attack, UV radiation as well as wounding.

Two main groups of PCs with antioxidant activity are flavonoids and phenolic acids (Nováková *et al*., 2009). Flavonoids are the largest group of naturally occurring phenolic compounds, which occurs in different plant parts both in free state and as glycosides **(**John *et al*., 2014). Peter *et al*. (1999) reported that the flavones and flavonols are the most widely distributed of all the phenolics. More than 6500 different flavonoid products have been characterized mainly for the fruits, colour of flowers and sometime the leaves but their real number may exceed 8000 (Ververidis *et al*., 2007).

The phenolic acids are derived from either benzoic or cinnamic acid (Nováková *et al*., 2009). Benzoic acid (gallic acid and vanillic acid) as well as cinnamic acid (caffeic acid, chlorogenic acid and ferulic acid) with esters can be found in herbal infusions, tea and wine (Atoui *et al*., 2005). Seal (2016) reported that phenolic acids play a potential protective role against different kinds of oxidative damaged diseases through consumption of fruits and vegetables.

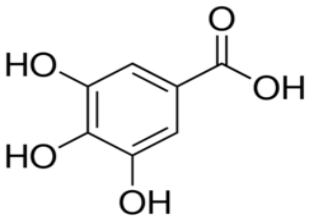
2.3.1 Gallic Acid

Gallic acid (GA) is a type of phenolic acid (3,4,5-trihydroxybenzoic acid) that has strong reducing character where in alkaline solution it becomes brown due to oxygen absorption (Condrat *et al*., 2009). For instance, the combination of its esters and glucose are components of tannins (Buzzini *et al*., 2008). GA is considered as a protection factor for plants against bacterial infections (antimicrobial activity), an antioxidant (strong reducing power) as well as a hydrogen carrier that participates in cellular redox systems (Zhang *et al*., 2009).

In nature, GA (Table 2.4) is found in oak bark, gall fly donuts on oak leaves and vegetal parts of some plants such as those from Plantae regnum (Condrat *et al*., 2009). Besides, GA also found in citrus fruits like *Phyllantus emblica*, (Amalaki), *Terminalia bellirica* (Bibhitaki), *Terminalia chebula* (Haritaki) and others (Kardani *et al*., 2013), is useful in common cold and fever, diuretic, laxative, liver tonic, hair tonic, alterative, antipyretic, anti-inflammatory (Kroes *et al*., 1992), digestive, refrigerant, restorative, stomachic as well as to prevent from peptic ulcer and dyspepsia.

GA and its derivative have antioxidant activities as well as a neuroprotective effects with free radical scavenging effects (Zhongbing *et al*., 2006 and Kubo *et al*., 2010). Choi (2005) and Kim (2009) reported that GA is used as an effective treatment for anorexia, bloating, gases, gastric tonus problems, urinary disease, gout, haemostatic, skin repairer and sedative.

Table 2.4: Characteristics of GA (Kardani *et al*., 2013)

Chemical Structure	
Structure Name	3,4,5-trihydroxybenzoic acid
Chemical Formula	$C_6H_2(OH)_3COOH$
Molecular Formula	$C_7H_6O_5$
Molar Mass	170.12 g/mol
Density	1.7 g/cm^3
Appearance	white, yellowish-white, pale fawn crystals

2.3.2 Quantification of Phenolic Compounds

Phenolic compounds can be quantified by total phenolic content (TPC) method. Schofield *et al*. (2001) indicated that polyphenols in plant extracts react with a specific redox reagents such as Folin-Ciocalteu reagent will form a blue complex that can be quantified by visible-light spectrophotometry. In general, the reaction provides accurate and specific data for several groups of phenolic compounds that change colour differently due to reaction kinetics (Folin and Ciocalteu, 1927) and differences in unit mass (Glasl, 1983). The concentration of TPC is expressed as mg of GA equivalents (GAE) per gram of extract (Sadat Moosavi *et al*., 2014).

Based on the TPC in the plant extracts, the selected parts can be divided into three ranges of GAE values. The lower (<10 mg GAE/g), middle (10-20 mg GAE/g) and higher (> 40 mg GAE/g) ranges of total phenolic compounds of plant extract dry weight (Maisuthisakul *et al*., 2007). Stanojević *et al*. (2009) reported that the phenolic components are most frequently found in methanol extracts from all plant species that contain GA, vanillic acid, chlorogenic acid, caffeic acid, umbelliferone and many more. Among these, umbelliferone is the most active one (Zidorn *et al*., 2005). The phenolic acids present in the plants are natural antioxidants (Oboh *et al*., 2008), anti-carcinogenic agent (Kampa *et al*., 2004), anti-inflammatory (Canadanovic-Brunet *et al*., 2006), antimicrobial agent (Stanojević *et al*., 2008) as well as cardioprotective agent (Caccetta *et al*., 2000).

2.4 Extraction of Phytochemicals

Extraction is the separation of chemical compounds (or secondary metabolites) of plant using selective solvents through standard procedures (Handa *et al*., 2008). The purpose of all extraction is to separate the soluble plant metabolites leaving behind the insoluble residue. The initial crude extracts of plants may contain complex mixture of many metabolites such as alkaloids, flavonoids, glycosides, phenolics and terpenoids (Azwanida, 2015). In addition, some of the initially obtained extracts may be ready for use as medical agents but some need further processing. Extraction from the plant is an empirical exercise in which different solvents are utilized under a variety of conditions such as time and temperature of extraction (Doughari, 2012). Extraction is influenced by the chemical nature of the compounds to be extracted, extraction method used, the storage time and conditions as well as the presence of interfering substances (Robbins, 2003).

2.4.1 Solvent Extraction

Solvent extraction is the most widely used extraction method where it involves the use of organic solvents. Various solvents have been used to extract different phytoconstituents. The plant parts (tubers, roots, stems, leaves, etc) are dried immediately either in an artificial environment at low temperature (50-60^{o}C) or dried preferably in shade in order to bring down the initial large moisture content to enable its prolonged storage life and stability (Doughari, 2012).

Maceration is a one of solvent extraction method which involved soaking plant materials (coarse or powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days (36 hours) with frequent agitation (Handa *et al*., 2008). The process intended to soften and break the plant's cell wall to release the soluble phytochemicals. After 3 days, the mixture is either pressed or strained by filtration, concentrated either in vacuum or by evaporation in order to concentrate the crude extracts. The choice of solvents will determine the type of compounds extracted from the samples (Azwanida, 2015). Farsi *et al*. (2013) indicated that the most common organic solvent used in solvent extraction is methanol because of its polarity can elute more flavonoid and phenolic compounds.

2.5 Quality Control of Microbial Growth

2.5.1 Food and Medicinal Material Radiation

The expert committee of WHO/IAEA/FAO approved three types of ionizing radiation to be used in food irradiation and other medicinal material (WHO, 1998). This includes:

- Gamma rays from radioisotopes cobalt-60 or cesium-137 are most often used in food irradiation application because of their high penetrating power and low cost.

- Electron beam generated from machine sources (accelerators) operated at or below an energy level of 10 MeV (million electron-volts).

- X-ray generating from machine sources operated at or below an energy level of 5 MeV.

The ultraviolet (UV) irradiation as a method of sterilization is not effective because of its low penetrating power (Sharma and Demicri, 2003). On the other hand infrared (IR) and microwave irradiation have proved to be a limited value because both of these methods are basically form of heating and consequently have the same disadvantages of using heat. Besides, Kovács and Keresztes (2002) reported that the quantum of IR light is small.

2.5.2 Radiation Doses

When an ionizing radiation emitted from a radioactive source penetrated into a medium (i.e. food) all or part of the radiation energy is absorbed by that medium. The quantity of energy absorbed by the medium is called the absorbed dose which is measured in "rad". The new unit used now is according to the international systems in the gray "Gy". It is equal to the absorption of 1 J/kg (IAEA, 1990). [1 Gy = 100 rad; 1 kGy = 100 krad; 10 kGy = 10^6 rad = 1 Mrad]. The amount of ionizing radiation energy absorbed in a unit of time is called the "dose rate". Radiation exposure was measured in Roentgen (R) and now is Coulomb (C) (WHO, 1998).

2.5.3 Gamma Irradiation

Gamma (γ) rays is the most energetic form of such electromagnetic radiation which having the energy level from 10 kilo electron-volts (keV) to several hundred keV (Borzouei *et al*., 2010). Therefore, gamma rays are more penetrating than other types of radiations such as alpha (α) and beta (β) rays (Kovács and Keresztes, 2002). Gamma rays are categorized as ionizing radiation because the radiations produce free radicals in the cell when they interact with atoms or molecules. These free radicals are emitted from different parts of atom from the nucleus (Ali *et al*., 2016).

Herbs and spices are exposed to a wide range of microbial contamination during their cultivation, distribution, harvest, processing, sale and storage (Seo *et al*., 2007). Sources of microbial contamination include the macro environment such as soil or plant in which the products are grown, dust, insects, faecal material and contaminated water (Chan, 2003). On the other hand, the microbial status of dried herbal material is not so much caused by secondary contamination during processing but it is primarily due to the fact that plants have their own microbial flora (Abou Donia, 2008).

Gamma irradiation has been extensively studied as a mean of reducing the microbial contamination of herbs and spices which was approved by the Codex Alimentarius Commission in 1983. Many experts agreed that radiation does not cause any toxicological changes or activation of irradiated food products. Therefore, decontamination by ionizing radiation is a safe, very effective, environmental clean and energy efficient process (Chmielewski and Migdal, 2005).

Several countries such as Argentina, Brazil, France, India, South Africa, USA and others have approved irradiation of herbs and spices for microbial

decontamination and insect's disinfectations. Gamma irradiation results in a much lower level of microbial contamination and it is the only effective treatment to meet standards set by processors operating under Hazard Analysis Critical Control Points (HACCP) or International Organization for Standardization (ISO) (WHO, 1994).

2.5.4 Application of Gamma Irradiation

Gamma Rays have been used as an effective way to maintain the quality of food products for many years (El-Mouhty *et al*., 2014). In addition, gamma radiation treatment of food can kill and sterile the insects as well as can prevent from reproduction of food-born parasites (Fan, 2005). Other than that, gamma irradiation is also used for food preservation (Abolhassani *et al*., 2013; Verde *et al*., 2013; Kim *et al*., 2014).

The effects of gamma irradiation on the plants pigments have not been investigated intensely (Ganapathi *et al*., 2008). However, there are evidences showed that the structure and concentration of pigments such as chlorophyll and carotene may be changed. Chlorophyll has an effect on the production of red blood cells while carotene acts as antioxidant for human.

In the last decade, gamma radiation treatment of many crops has drawn the attention as a new and rapid method to improve both qualitative and quantitative characters (Desai and Rao, 2014). Gamma irradiation has also been widely applied in medicine where biological effects induced by a counter intuitive that switch over from low doses stimulation to high doses inhibition respectively (Charbaji and Nabulsi, 1999). Low doses of ionizing radiation on plants and microorganisms are manifested as cell proliferation accelerated, germination rate, enzyme activity, cell growth, crop yields and stress resistance (Chakravarthy and Sen, 2001).

In agriculture, the irradiation of seeds may affect the genetic make-up of the plant development where variability enable plant breeders to select new genotypes with improved characteristics including precocity, quality, grain yield and salinity tolerance (Ashraf, 2003). Besides, ionizing radiations are also used to sterilize some agricultural products to increase their conservation time or to reduce pathogen propagation (Melki and Salami, 2008). The irradiation of wheat seeds reduced shoots and root lengths upon germination (Chaudhuri, 2002).

In biomaterial, gamma irradiation can be a convenient tool for modification of polymer materials through grafting, degradation techniques and cross-linking (Singh *et al*., 2010). Furthermore, it has been suggested as a rapid and convenient modification technique which breaks large molecules into smaller fragments as well as capable of cleaving glycosidic linkages (Kang *et al*., 1999; Yu and Wang, 2007). As a result, the chemical bonds of starch can be hydrolyzed by gamma irradiation leading to degradation of the polymeric chain and may be useful in applications for building, paper and textile materials (Kang *et al*., 1999).

2.6 Microbial Limit Test (MLT)

The MLT is designed to perform both qualitative and quantitative estimations of specific viable microorganisms present in samples. The tests include total viable count (bacteria and fungi). The term ‘growth’ is used to identify the presence and presumed proliferation of viable microorganism. Either the samples have antimicrobial activity or include with antimicrobial substances, these antimicrobial properties must be eliminated by dilution, filtration, inactivation, neutralization or other appropriate means (Iqbal *et al.*, 2012). The tests for samples prepared must be conducted by mixing multiple portions randomly chosen from individual products or

ingredients. Utmost care should be taken while performing the tests in order to avoid any microbial contamination from the outside surrounding. Due attention also must be paid for the prevention of biohazard and quality control effectiveness.

2.6.1 Total Viable Aerobic Count

The test is to determine mesophilic bacteria and fungi that grow under aerobic conditions (presence of oxygen). Some bacteria such as psychrophilic, basophilic, thermophilic and anaerobic which require specific ingredients for growth may give a negative result even though they exist in a significant number (Aquino, 2011). Membrane filtration, pour plate, spread plate and serial dilution (most probable number of method) are four available methods for this test. Incubation temperature and different media are required for the growth of bacteria and fungi (moulds and yeasts). The serial dilution method is only applicable for bacteria.

2.6.2 Buffer Solutions and Media

Buffer solutions such as phosphate buffer (pH 7.1-7.3) and sodium chloride-peptone buffer (pH 6.9-7.1) as well as media listed in Table 2.5 are commonly used for MLT. Other media may be used if they have similar nutritive ingredients, selectivity and growth-promoting ability toward the microorganism to be tested.

Table 2.5: List of Media for MLT (British Pharmacopoeia, 2016)

Media	pH
Soybean-casein digest agar medium	7.1 – 7.3
Fluid soybean-casein digest medium	7.1 – 7.5
Antibiotics-added Sabouraud glucose agar medium	5.4 – 5.8
Antibiotics- added potato-dextrose agar medium	5.4 – 5.8
Antibiotics-added GP (glucose-peptone) agar medium	5.6 – 5.8
Fluid lactose broth medium	6.7 – 7.1
BGLB (brilliant green lactose bile) medium	7.0 – 7.4
MacConkey agar medium	6.9 – 7.3
EBM (Eosin-methylene blue) agar medium	6.9 – 7.3

2.7 Stability Study

The stability of extracts obtained from various plant materials are essential to ensure the efficacy, safety and quality of the finished product in the market (Gafner and Bergeron, 2005). The purpose of stability study is to establish a shelf life for the medicinal product and recommended storage conditions **(**SADC, 2004).

The stability of finished pharmaceutical or food products depends on environmental factors and product-related factors (Kopp, 2006). Environmental factors such as ambient temperature, humidity and light while product-related factors include the chemical and physical properties of the active substance and pharmaceutical excipients, the dosage form and its composition, the manufacturing process, the nature of the container-closure system as well as the properties of the packaging materials.

The frequency of testing for long-term studies should be sufficient to establish the stability profile of the product. According to the International Conference on Harmonisation (2003), the frequency of testing at the long-term storage condition should normally be every 3 months over the first year, every 6 months over the second year and annually thereafter. Meanwhile, at the accelerated storage condition, a minimum of three time points including the initial and final time points (0, 3 and 6 months) from a 6-months study. As a result of significant change at the accelerated storage condition (for testing intermediate storage condition), a minimum of four time points including the initial and final time points (0, 6, 9 and 12 months) from a 12 months study. The storage conditions for long term, intermediate and accelerated are shown in Table 2.6.

Table 2.6: Storage Condition for Stability Study (WHO, 2009)

Study	Storage Conditions	Minimum of Time Period
Long Term	$25^{o}C \pm 2^{o}C$; 60% RH ± 5% RH $30^{o}C \pm 2^{o}C$; 65% RH ± 5% RH $30^{o}C \pm 2^{o}C$; 75% RH ± 5% RH	12 Months
Intermediate	$30^{o}C \pm 2^{o}C$; 65% RH ± 5% RH	6 Months
Accelerated	$40^{o}C \pm 2^{o}C$; 75% RH ± 5% RH	6 Months

2.8 Instrumental Techniques in Phytochemical Analysis

2.8.1 High Performance Liquid Chromatography (HPLC)

Liquid Chromatography (LC) is the most widely used of all of the analytical separation techniques. This is because of its sensitivity, its ready adaptability to accurate quantitative determinations, its ease of automation, its suitability for separating non-volatile species or thermally fragile ones as well as its widespread applicability to substances that are important to industry, many fields of science and the public (Holler *et al*., 2007). Such materials include amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, terpenoids, pesticides, antibiotics, steroids, metal-organic species and variety of inorganic substances.

The principle of HPLC is similar to thin layer chromatography (TLC) whereas in HPLC, the stationary phase (silica-packed column, C8 or C18) is non-polar while mobile phase is polar. HPLC system consists of detector, pump (binary or quaternary), autosampler and data workstation. Binary pump is able to deliver only two different types of solvent at the same time while quaternary pump able to deliver four different types of solvent at the same time. A few detectors such as diode array detector (DAD), ultraviolet (UV), flame ionization detector (FID), refractive index detector (RID), cathodoluminescence (CL) and others can provide both qualitative and quantitative applications (Wolfender, 2009).

UV detector is commonly used in natural product research and development due to its sensitivity, versatility, linearity, and reliability. Most of natural product components are able to absorb UV light within 200 -550 nm. For instance, one or more double bonds or weak chromophores such as saponin can be detected at wavelength 203 nm (Vial and Jardy, 1999) while phenolic compound such as gallic acid can be detected at wavelength 272 nm (Kardani *et al*., 2013). Sensitivity and selectivity of method development such as standardization, detection, quantification, fingerprint and screening required a small amount of sample analysed by HPLC.

2.8.1(a) Qualitative Analysis

Qualitative analylis is the scientific study of data that can be observed and identified but cannot measured. It involves running a standard that contains the target analytes. The most common parameter for compound identification is its retention time (the time it takes for that specific compound to elute from the column after injection). Compound identification is based on the chemical structure, molecular weight or some other molecular parameter.

2.8.1(b) Quantitative Analysis

This is a scientific study of data that determine the amount of a compound that can be measured. A sample with a known amount of the compound of interest is determined by measuring its peak height or peak area in order to make a quantitative assessment of the compound. In many cases, there is a linear relationship between the height or area and the amount of sample. The data must be acquired and processed under identical conditions in order to obtain a valid comparison for the unknown sample response to the known standard.