Quantification of Total Phenol and Flavonoid content, In vitro Investigation of Antioxidant Activity and Cytotoxic Effect of the Seed Extract of *Piper nigrum* Linn (White pepper)

A project submitted by

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Dedicated to my parents to whom I owe my achievements.

May Almighty keep you as well as you have kept me.

Certification Statement

This is to certify that, this project titled ‘Quantification of Total Phenol and Flavonoid content, In vitro Investigation of Antioxidant Activity and Cytotoxic Effect of the Seed Extract of *Piper nigrum* Linn (White pepper)’submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Ridwan Islam, Senior Lecturer, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the Supervisor

____

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Abstract

Piper nigrum Linn or pepper as more commonly known, is an essential everyday spice. Current study endeavours to assess the healing and medicinal properties that *Piper nigrum* exhibits, its role in traditional medicine and its application today. It is reported to possess antioxidant qualities that neutralize harmful "free radicals" in our body, many arguments are still being pointed towards its actual affectivity. In the present study, *in vitro* antioxidant activity of the seed extract of white pepper was evaluated by DPPH (2,2-diphenyl-1-picrylhydraziyl) free radical scavenging assay and total flavonoid and phenol content determination. In order to determine the cytotoxic effect of the extract, brine shrimp lethality test has been carried out where the LC_{50} value were determined and compared with the standard like vincristine sulphate. Our investigation indicates that the various fractions of the seed extract of white pepper contain high amount of phenol and flavonoid content with significant cytotoxic effect which may justify the traditional use of pepper and makes it as potential natural source in drug discovery and development.

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Abbreviations

ROS - Reactive Oxygen Species

SOD - Superoxide dismutase

CME - Crude Methanolic Extract

CCE - Crude Chloroform Extract

CNE - Crude n-hexane Extract

PPE - Petroleum Ether Extract

DPPH - 1, 1-diphenyl-2-picrylhydrazyl

BHT - Butyl-1- hydroxy toluene

ASA - Ascorbic Acid

DMSO - Dimethylsulphoxide

NaCl - Sodium Chloride

GAE - Gallic Acid Extract

STD - Standard Deviation

VS - VincristineSulphate

TPC -Total Phenol Content

TFC -Total Flavonoid content

Chapter One: Introduction

1.1 Rationale and objective of the present study

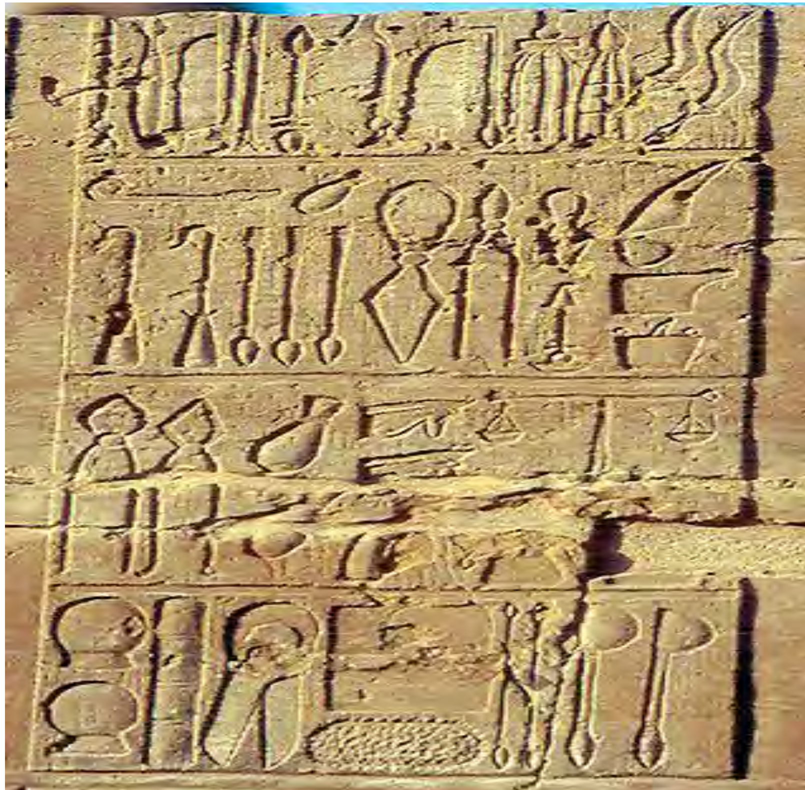
In order to improve the quality of life, consumption of medicinal herbs has tremendously increased over a past decade as an alternative approaches to maintain a good health. Plants of medicinal value have always been used for treating human diseases from ancient times. Ordinarily, when natural resistance of the organism are inundated by an enormous formation of ROS (reactive oxygen species) a situation of 'oxidative stress' occurs where macromolecules (proteins, lipids, and nucleic acids) may suffer oxidative deterioration, causing tissue damage, several human chronic diseases, cardiovascular diseases, mutagenesis and cancer, several neurodegenerative disorders, and the aging process (Fontenot, 2011).

"There is no question in my mind that herbal medicine will continue to flourish and eventually become integrated into our material medica. Considering the past, it is undeniable that an accord of development has already been made. We now looking forward to the future with eager anticipation and great expectations."-Tylor V.E. *Herbal medicine: from the past to the future,* p452

Medicine has reached heights far surpassing even the imagination of our forefathers. Such is the miracle of modern medicine that complex research subjects such as stem cell research is no more a prospect but a thriving reality. However, with all these advances we still find fatal diseases such as cancer to be on the rise, especially in third world countries. In a report it is stated that by 2030, an estimated 13 percent of all deaths in Bangladesh will be related to cancer with 200,000 new cases reported every year, up from 7.5% in 2005 (bdnews24, 2015-12-15). These statistics shine light on issues that contradict the above mentioned advances as the rate of fatal diseases are supposed to go down as we move forward, not up. This is merely only one example from a list of many and studies have shown that the side effects of

synthesized food and certain medicines have created a platform for these diseases to spread. One such product is synthesized antioxidants which a majority of scholars believe to be more harmful than helpful for humans (Hasslberger, 2007).

Herbalism is the oldest medical practice, dating its roots as far back as 33rd century BC Egypt. These were recorded in stone and are now called the “Egyptian medical papyri” as shown in the image bellow-



Marry, Austin (January 21, 2004). Ancient Egyptian Medical Papyri. *Eircom limited*. Retrieved from https://en.wikipedia.org/wiki/Egyptian_medical_papyri

Today we have managed to achieve great advances by using technology to better understand the knowledge we have accumulated over thousands of years. We are now able to extract the

counteractive phyto-elements produced inside the plant's personal laboratory to aid us in keeping our bodies healthy. There are several advantages of producing medicines through plants and to point out one would definitely be their sheer variety of species. According to Botanic Gardens Conservation International (bgci.org), in the first ever checklist of the world's plants created in 2010 lists around 3,50,000 accepted varieties of plants with over 240,000 still to be confirmed. All of these plants produce an assortment of chemical compounds that it uses in different scenarios. Plants are not animated objects so they rely on these chemicals to shield them against bacterial infestation and decay. We have already isolated about 12,000 of these chemicals and is only estimated to be a mere 10% of the chemicals plants produce. For the sake of this study, however, we will only be looking at a specific sub-study of herbology like spices and more specifically on pepper-white pepper.

Therefore, the rationale behind choosing such experimental work is to develop herbal medicine along with potential antioxidant from natural sources like spices. The investigational plant part which has been used in the present study are the seeds of unripe fruits of white pepper or *Piper nigrum Linn*.

The present study insights into the phytochemical and biological investigations of seeds extract of *P. nigrum Linn* which includes:

- Extraction of the dried powder of seeds of *P. nigrum* with methanol by cold extraction method, Fractionation of methanolic extract and separation of methanol, n-hexane & chloroform fraction.
- Determination of total phenolic and flavonoid content of methanolic, chloroform, and n-hexane extract by Folin-Ciocalteu regent and aluminium chloride respectively.
- In vitro assessment of all the three fractions of crude extract of white pepper for antioxidant activity by DPPH radical scavanging assay.
- Brine shrimp lethality test to determine the cytotoxic effects of all the fractions of white pepper extract.

1.2Scenario of medicinal spice-pepper in Bangladesh

There's no specific difference between herbs and spice. Herbs basically render the leaves whereas spice includes the fruits, flowers, seeds or barks of plant origin. Since ancient times, humankind have been crushing, pinching, dashing and tossing spices in pots and pans. Spices not only mask the odor and taste, it also enhance the flavor, prolongs the freshness of the food as well. Nature itself has provided plenty of nutrients which are beneficial for our health. Many spices are high in volatile oils which may aid digestion and also act as a relaxant for our nervous system. Others may rich in antioxidants that offer protection from DNA damage as well as enhance the activity of body's own antioxidant enzymes. Spices such as pepper contain plant compounds that even in small amounts may bring us health and vitality while creating the depth of flavor preventing spoilage in our food (Purseglove, 1981).

Pepper is considered as the `king of spices'. It is the most traded spice in the world. It is a tiny spice but very strong and effective. Pepper is the fruit of *Piper nigrum* (Piperaceae). *P.nigrum* depending upon its process of preparation and modification these may results in white, red, green or black peppercorns all of which has unique tastes (The Editors of Encyclopaedia Britannica, 2016). In our present study, the main investigation will be upon white pepper as very few experiments have been done with it.

Native to India, pepper has played very important role throughout history, a prized spice since ancient times. Once used as currency and presented to the gods as sacred offering it is fortunate that this most popular of spices is available throughout the year. But only in modern times we are able to understand the benefits of this wonderful spice, not only as a culinary essential but an investment in medical research (Shanmugapriya, 2012).

1.2.1 Importance of pepper in the past

Since pepper is the spice we will be working with, it is necessary to understand its value in history. The sole use of pepper is in the seasoning of food owing to its aroma and pungency. In traditional medicines, this spice is also reported to have digestive power, to improve

appetite, and to cure cold, cough, dyspepsia, diseases of the larynx-pharynx, intermittent fever, stomachache, diarrhoea, louse and piles. In folk medicine, pepper is also used against epilepsy and snake bite. In 5th century Syriac Book of Medicines prescribes pepper (or perhaps long pepper) for such illnesses as constipation, dysentery, earache, gangrene, heart disease, herniation, hoarseness, digestive problems, insect bites, insomnia, pain at the joints, liver, lung diseases,sunburn, tooth decay, and toothaches. Pepper root, in the form of ghees, powders, enemas and balms, is a folk remedy for abdominal tumors (Kadamet al., 2013).

1.3 Necessity of potential antioxidant of natural source

1.3.1 Understanding antioxidants

Natural antioxidants are found in lots of plants and numerous foods and spices we eat every day. They help fight oxidation, a typical reaction that happens in the body each day. At the point when there are disturbances in the natural oxidation process, exceptionally unstable and possibly harming atoms called free radicals (further explained below) are formed. Oxygen triggers the arrangement of these devastatingly destructive little chemicals and if left uncontrolled, they can harm cells in the body. It's much like the reaction that makes rust on a bike or turns the surface of a cut apple dark. Antioxidants work by donating an electron to a molecule that has been compromised by oxidation, bringing it back into a state of proper function. Having been used up in this process, anti-oxidant molecules are renewed by taking an electron from an alternative antioxidant or is modified into building matter such as collagen used in repairing tissue matter (Berkeleywellness, 2015). On the contrary, synthesized versions of anti-oxidants cannot be used in this manner after they lose their electrons. After being "spent" they tend to become dangerous byproducts that generate further pressure on the oxidative load of the entity. Antioxidant deficiency is linked with major diseases such as Alzheimer's, cancer, Parkinson's and more (Y. Feng & X. Wang, 2012).

1.3.2 Antioxidant as protection

A highly evolved and intricate defense mechanism using antioxidants has been created in humans to fight against reactive oxygen species and protect our bodies. It employs a selection of components, both endogenous and exogenous, that work interactively to counteract free radicals. Some of the most extensively researched dietary antioxidants are

vitamins E and C. Vitamin C is able to counteract ROS in its liquid phase before lipid peroxidation and is thought to be a very potent, if not most important water-soluble antioxidant. Vitamin E is the most efficient chain-breaking antioxidant inside the cell membrane.Vitamin C is known to help reproduce vitamin E. Fruits and vegetables contain a big supply of vitamin C while whole grains and high quality vegetable oils are major sources of vitamin E (Wang & Quinn, 1999).

1.3.3 Endogenous antioxidants

In addition to dietary antioxidants, the body depends on a few endogenous barrier systems to secure itself against free radical-affected cell damage. The antioxidant enzymes – catalase, glutathione peroxidase and superoxide dismutase (SOD) – metabolize dangerous intermediates and require micronutrient cofactors, for example, selenium, iron and zinc for ideal catalytic activity. It has been proposed that a lacking dietary admission of these follow minerals might hinder the performance of this defense mechanism (Fontenot, 2011).

1.3.4 Free radicals

A free radical is defined to be any atom with a minimum of one unpaired electron in the outer shell and capable of existing on its own. Formation of free radicals occur easily when a covalent bond breaks up and one electron is leftover with the newly created atoms. Their highly reactive qualities come from the free electron in the outer shell. The fact that they are highly reactive means that they can react with most molecules in its vicinity. This includes proteins, lipids, carbohydrates and DNA. Hence, free radicals bond with the nearest available molecule, "stealing" its electron. When the "attacked" molecule drops an electron and itself becomes a free radical, creating a chain reaction. The process can cascade to dangerous heights which may result in the disruption of living cells (Hasslberger, 2007).

Now that we somewhat understand the crisis created by free radicals in our system we need to look at methods of countering them. As stated, synthesized antioxidants have little to no effect in countering free radicals. In fact, when they are used up, they themselves become a harmful agent in our bodies, creating a most dangerous medicinal paradox. Hence, we turn our interests to natural means, going back to the ‘roots’ of medicine as we know it today. Nowadays, it has become a popular misconception that herbal medicine is medieval, a

primitive means of treatment. What we fail to realize is that through millions of years of evolution our bodies have become the perfect surviving tools nature has to offer. Its means of survival comes from nature and over millions of years have become accustomed to its products. Everything we need to be healthy can be found in nature. Thus, plants and natural foods have been used in medical purposes before history was accounted.

1.4 Cytotoxicity Study

Toxicology takes its roots in the use of of assassination, bloodshed, crime and suicides. Largely popular in pre-biblical Greece, a well-known example is that Nicandar of Colophon (185-135 BC) experimenting with poisons on criminals of the Bynthian Kingdom. The extensive use of poison in this manner deemed in crucial to devise treatments and Maimmonides wrote "Poisons and their antidotes" in the 12th century. (John Timbrell, Introduction to Toxicology, Third edition, p3-4)

However, it is not till recent times that toxicology made major strides in scientific pursuits, more importantly cytotoxicity - the degree to which an agent has specific destructive action on certain cells. This study gives credit to 16th century scientist and chemist, Paracelsus who said that,

a poison is only potent after a certain dose.

One of the most widely used modes of testing cytotoxicity is the brine shrimp lethality bioassay. It gained recognition due to its rapid and comprehensive nature for the bioactive compound of natural and synthetic origin. It allows for the bioactivity testing of natural product extracts, fractions as well as the pure compounds. This assay helps in determining pharmacological activities like antimicrobial, anti-viral, pesticidaland anti-tumor activities of bioactive compounds of natural and synthetic origin (Pisutthanan, 2004).

Chapter Two:

Plant Description

& Literature Review

2.1 The Piperacea Family

The Piperaceaefamily is a large family of blooming plants. This family is also recognized as The Pepper Family.This group consists of approximately 3600 currently accepted species in 13 genera. The two main genera are *Piper* (2000 species) and *Peperomia*(1600 species), where the maximum kind of peppers can be found (Kato & Furlan, 2007). The species of the pepper family can be shrubs,herbs and small trees. The distribution of Piperaceae family is best described as pantropical. The most acclaimed species of Piperaceae family are known as *Piper nigrum*. It yields most peppercorns which are used as spices. This family mainly includes black pepper and white pepper although its relatives may include many other spices(Yuncker, 1958). Depending upon the genetic research on recent ways of categorizing plants, they have been classified into three sub families. They are-

- Subfamily : Verhuellioideae Samain and WankeVerhuellia Miquel 1843
  (three species)
- Subfamily : Zippelioideae Samain and Wanke ZippelliaBlume 1830
  (one species), ManekiaTrelease 1927 (six species)
- Subfamily : PiperoideaeAmott
  Piper Linnaeus 1753 (2000 species)
  Peperomia Pavon and Ruiz 1794(1600species)

They contain about five genera among them. The Piperoideae contains about two genera among which one is Peperomia and the other one is Piper. The Verhuelloideae consist of the single genus Verhuellia. The rest two genera of the Zippelioideaeare Manekia and Zippelia (The Editors of Encyclopaedia Britannica, 2016).

The Piperoideae subfamily is considered as the maximal amidst the three subfamilies so far. Among them, the two most plentiful genera are Peperomia and Piper. There is around 2000

Piper species and 1600-1800 Peperomia species. Conversely, altogether only 10 species lie under the rest of the genera (The Plant Profiler, 2015).

2.2 Charecteristics

It is not easy to define the Piperaceae family. Most of the prevalent species of this family are herbs and many of them take the articulationof shrubs, small trees and lianas. Some characteristics are common among the members of Piperaceaefamily.All the constituents of this family carry androgynous flowers in the cast of packed spikes. Petals or sepals are missing in these flowers. They are tiny and radially symmetrical. But they do have small modifies leaves associated with the plant reproductive structures. The leaves of Piperaceae family are usually soft, succulent or fleshy. They got simple shapes but the entire edges are often heart shaped. They can be curled, alternate or rolled in organization. The leaves may grow along the stem or base of the plant. A noticeable aroma is found when they are crushed. The fruits of the Piperaceae family comprises single seed inherited in drupe like fruit. Each seed has gritty perisperms along with one little embryo (Kadamet al., 2013).

Table 2.1 The plants belonging to the Piperaceae family in Bangladesh

Germplasm Resources Information Network (GRIN), 2007 - 2011

Scientific Name	Local Name
Piper betle	Supari
Piper retrofractum	Choi
Peperomiapellucida	Luchipata
Piper peepuloides	Pipul

2.3 INVESTIGATED PLANT :*Pipernigrum Linn* (White pepper)

NODC Taxonomic Code, database, 1996

Table 2.2: Taxonomical classification

KINGDOM	Plantae
SUBKINGDOM	Viridiplantae
SUPER DIVISION	Embryophyta
DIVISION	Tracheophyta
CLASS	Magnoliopsida
SUBCLASS	Magnolianae
ORDER	Piperales
FAMILY	Piperaceae
GENUS	Piper
SPECIES	*P. nigrum*

2.4 Vernacular Name:

Table 2.3 Vernacular Name of White Pepper

Bengali Name	Shada golmorich
Hindi Name	Safed golmorich
Punjabi	Safed golmorich
English Name	White pepper

Arabic	Fulfulaswad
Finnish	Pippuri
German	Pfeffer
Italian	Peppa
Japanese	Peppapepaa

(L.D. Kapoor, CRCHandbook of Ayurvedic medicinal plants)

2.5 Geographical Distribution

Before 16th century, pepper was grown in Java, Sumatra,Sunda, Malaysia, Madagascar and everywhere in Southeast Asia. Pepper is native to South Asia and Southeast Asia. Now pepper is cultivated in tropical areas around the world including China, West Indies, Malay Penninsula, Malay Archipelago, Siam, Malabar etc (Botanical-online, 1999-2016).

2.6 Plant Description

Piper nigrum Linn (white pepper) is a flowering vine of Piperaceae family. It is mainly harvested for its fruits which are termed as peppercorns. The dehydrated fruits are used and preserved as spices. When it is dried, is approximately 5mm in diameter. The colour is dark red when it is fully matured and like all drupes contains a single seed. The ground pepper or pepper corns derived from them are simply denoted as pepper. The cooked and dried unripe fruits are considered as black pepper and more precisely the ripe fruit seeds are known as white pepper.The pepper plant is an amaranthanperennial affixed to trees through aeriform roots. It is an equatorial plant and cultivated in hot moist areas with high rainfall (Purseglove,1981)

Root : A shallow root system is found in pepper plants. There are usually some primary side along plants which have the ability to pass through the soil to the depth of 2m.

Stems : There are about three types of stems in pepper plant. e.g

- The primary stem- it forms the main stem from which others may develop.
- The secondary stem- These stems are circular, lengthy shoots with extended internodes. They can jump toa ample height and afterwards slumped downwards.

- The tertiary stem- these type of stems are short, got sturdier branches which may profused in line with the horizon from the axils of both primary and secondary runners.

Leaves : The leaves of pepper plant are dark green on the upper and whitish green on the under side. They are about 8-24 inches long and 12.5 inches wide. The leaves are arranged alternatively and pointed at the tip.

Flowers : The flowers of this family are mostly white and bisexual.The flowers assemble in ovoid spikes that further may develop into clusters. The flowers and clusters always bloomon tertiary wood and on opposite side of leaves.

Fruits : Nine months after flowering, the fruits appear which are like round berries. Their diameter are about 4-10 mm and may coincidice in elongated clusters. The seeds are of 3-6 mm encapsulated in each berry and are spherical in shape.

Seeds: The largest volume of the fruit is occupied by the seeds. They are approximately 3-4 mm in diameter (Meghwal & Goswami, 2012).

2.7 Soil and environmental requirements for growth of plants

- Pepper plant cannot be cultivated in places, the temperature may fall under 12 degree Celsius. A moderate climate is suitable for the growth of pepper plant.
- About 2000 mm rain annually is required for the growth of pepper plants. As rainfall is needed for the cultivation pepper plant so in South Africa the rainfall must be subsidized with irrigation.
- The drainage system must be good for the prevention of root rot.
- The soil must have a good structure with water holding capacity. The pH should be from 5.5 to 6.0. High humus content is advantageous for the plant as pepper plants react well to organic fertilization (Vijayalaxmi& Roy, 2013).

2.8 Folk medicinal uses

Historically pepper was used both as a spice and seasoning .The Folk medicinal uses of white pepper are almost same as Black pepper. Various medicinal uses are as follows:

- In the Philippines, pepper has been used as an excitant and as an agent (rubefacient) for causing redness or irritation on the skin.
- It was also used to treat alopecia and skin diseases by applying it externally as a patch.
- White pepper feature as remedies in Ayurveda, Siddha &Unani medicine.
- They are used in heart-burn, herpes,vertigo, paralytic & arthritic disorder, & chronic rheumatism
- Used for obstinatingacidosis and small fevers
- The Infusion has also been used as gargle to relieve from itchy throats.
- Toasted berries are helpful for stopping vomiting related to cholera.
- The juice of the boiled leaves of *P. nigrum* were used as remedy for scabies.
- White pepper, either powder or decoction is widely used in tradition India medicine to relief toothache.
- A mixture of white pepper honey and ginger is regarded as abortifacient.In India, Pepper were merely used as cough suppressant, malarial fever reducer, dyspepsia, constipation, dental carries and toothaches (Vijayalaxmi & Roy, 2013).

2.9 Literature Review

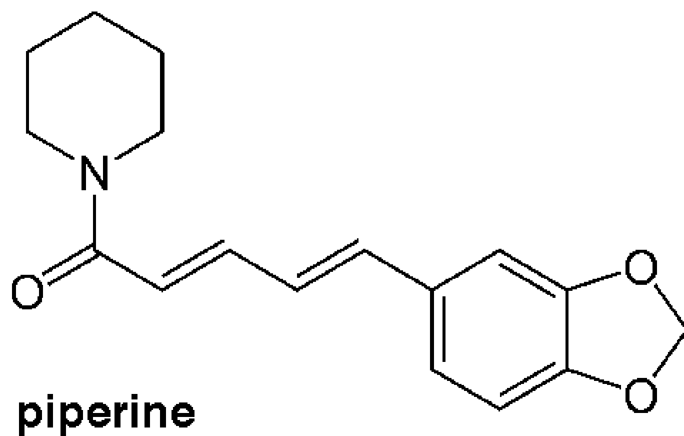
When peppercorns are consumed, it is likely that they are hydrolyzed in the gut, liberating bound polyphenols. Hydrolyzed (total polyphenol) extracts of both white and black pepper had significantly higher polyphenol concentrations than the non-hydrolyzed (free phenol). The high polyphenol concentration along with the in vitro radical scavenging activity of the peppercorn extracts proves their contribution in nutrition. Peppercorn could therefore be an useful source of antioxidants in preventing oxidative stress associated disorders. Conversely, in vivo studies need to be implied in order to confirm its potential (Gabriel et al., 2006).

Chemical investigations revealed that b-caryophyllene is a major component of WPEO. It also justified that, in both oleoresins such as in WPNH and in WPET, piperine can be considered as a primary component. WPEO and both oleoresins of white pepper were found to be effective antioxidants. In addition they show remarkable antimicrobial activity. So it is

inferred that if applied to food products, they could be used as natural food preservatives (Sunitaet al., 2013)

White pepper extract affects growth rate of *Streptococcus mutans in vitro* and also prove its antibacterial properties. There is a very strong negative correlation between white pepper extract and the growth of *Streptococcusmutans*. It has been proved that, the more the concentration of the white pepper extract, the lower growth rate of *Streptococcus mutans* (Yona et al., 2013).

As come from same plants phytochemical composition of white pepper, they resemble to black pepper but containing more starch and less ash. Previous literature shows that it contains various classes of chemical compounds like volatile oil, resin, starch, protein, alkaloids, flavonoids, lipid etc. A major constituent of white pepper is Piperine. Chavicine is the isomer of piperine.Piperine not responsible for the aroma of the white pepper but piperine imparts pungency to the white pepper (Kadamet al., 2013).



Volatile oil of White pepper

The following table2.4 and table 2.5 represents the list of essential oil and oleoresins that are present in white pepper (Singhet al., 2013).

Table 2.4 Volatile oil content of white pepper as follows:

Volatile oil	**Percent (%)**
α-Pinene	2.5
Camphene	Trace
β -Pinene	7.3
Myrcene	0.9
α -Phellandrene	0.4
3-Carene	0.3
α -Terpinene	0.2
α -Thujene	0.8
p-Cymene	0.2
Limonene	11.9
Sabinene	12.6

Table 2.5 Oleoresin of white pepper

Oleoresin	**Percent (%)**
β-Caryophyllene	0.2
β -Bisabolene	0.1
Cadinene	0.1
Caryophyllene oxide	trace
Torreyol	0.2
Pellitorin	0.4
Piperlonguminine	2.2
Piperolein B	6.6
Piperettine	0.4
Sitosterol	0.7
Dehydropipernonaline	2.0

Chapter Three: Methodology

3.1 Chemical work

The following processes roughly show the chemical work done on the *Piper nigrum Linn* seeds:

1. Proper collection and identification of the plant sample.
2. Preparation of plant sample.
3. Extraction of plant part with methanol, chloroform and n-hexane separately.
4. Solvent evaporation that will yield crude methanolic extract (CME),crude chloroform extract (CCE) and crude n-hexane extract (CNE).
5. Chemical tests or determination of total phenol content and flavonoid content of the three partitionates- crude methanolic extract (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE)
6. Antioxidant assay of the all the fractions of the extract.

3.1.1 Collection and Identification of the Plant Sample:

The *Piper nigrum Linn* seeds were taken from the local market in Dhaka city, Bangladesh during September, 2015.

3.1.2 Preparation of Plant Samples:

After collecting, the seeds were comprehensively cleaned with tap water and were dried out under sunlight for a few days. They were further dried in an oven for 24 hours at a low temperature to help with grinding. These samples were made into a powder in a grinding mill. The coarse powder was then stored in an air tight container and kept in cool and dry place.

3.1.3 Extraction and Solvent Evaporation:

The powdered plant materials were extracted by cold extraction process. Crushed plant material (seeds) were taken in a reagent bottle and soaked in methanol (500ml), chloroform(500ml) and n-hexane (500ml) separately. The content was kept in a bottle for a period of seven days, occasionally being stirred and shaken. The entire mix was then filtered through cotton and then through Whitman no.1 filter paper and then was made concentrated

with a rotary evaporator under reduced pressure at elevated temperature to get crude extract known as crude methanolic extract (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE).

3.1.4 Chemical tests (total phenolic and flavonoid contents) of CME, CCE, CNE:

3.1.4.1Determination of Total Phenolics:

Plant polyphenols, a diverse group of phenol compounds have a good structural chemistry for free radical hunting (Ahmed et al., 2014).

Principle:(Ahmed et al., 2014)

The content of all the phenolic compounds of the various extracts in the plant was found using Folin–Ciocalteu Reagent (FCR). The complete chemical components of the FCR is not known but is thought to have heteropolyphosphotungstates – molybdates. A series of reversible one or two electron reduction reactions result in a blue species. Molybdenum is understood to be easily reduced in the complex reaction that happens between reductants and Mo(VI)

Reagents and chemicals

- Folin-ciocalteu reagent
- Sodium carbonate
- Methanol
- Gallic acid
- Distilled water

Experimental procedure:

0.5 mlof plant extract was placed in a test tube containing 2.5 ml of Folin-ciocalteu that was diluted 10 times with water was then added to the mixture. 2.5 ml of Sodium Carbonate was added and the solution was incubated for 20 minutes at 24°C temperature. Every reagents should be prepared freshly.

The absorbance was then fixed at 760nm using a spectrometer against a blank. The standard blank solution had all the regents with the exceptions of plant extract or standard solutions. The below equations were used to calculate the content of phenolic compounds in plant extract and in polyphenolic fractions in GAE:

$C = (c \times V)/m$

where,

C= the total content of phenolic compounds,mg/g plant extract in GAE

c =concentration of gallic acid obtained from the curve (mg/ml)

V=the volume of the sample solution (ml)

m=weight of the sample (g)

All samples were analyzed trice and averaged results are taken.

3.1.4.2 Determination of Total Flavonoids

Principle (Kadam et al., 2013)

The content total flavonoids in different fractionates of plant extract was determined by the well known aluminum chloride colorimetric method. In this method aluminum chloride forms complex with hydroxyl groups of flavonoids present in the samples. This complex has the maximum absorbance at 420 nm (Kadam et al., 2013).

Reagents and chemicals

- Aluminium chloride
- Sodium nitrite
- Catechin as standard
- Methanol
- Distilled water

Experimental Procedure:

0.5ml of plant extract or standard were taken in test tubes with 2.5 ml of distilled water. Then 0.15 ml of 5% sodium nitrite were added and after 5 minutes 0.3 ml of 10% aluminium chloride were added following the addition of 0.55 ml distilled water to the

mixtures. Again, after 5 minutes- about 1ml of 0.001M sodium hydroxide was added. All the reagents must be freshly prepared.

The absorbance was then fixed at 760nm using a UV-spectrometer against a blank. The standard blank solution had all the regents with the exceptions of plant extract or standard solutions. The below equations were used to calculate the content of flavonoid compounds in plant extract and in standard catechin. The total content of flavonoids of the partitionates were expressed as catechin equivalents after calculation using the following equation :

$C = (c \times V)/m$

where,

C= total flavonoid contents,mg/g of plant extract in catechin extract

c =concentration of catechin obtained from the curve (mg/ml)

V=the volume of the sample solution (ml)

m=weight of the sample (g)

Similar to determination of phenol content, here again all the samples were analyzed trice and averaged results are taken.

3.2.1Antioxidant Assays:

3.2.1.2 DPPH (1, 1-diphenyl-2-picrylhydrazyl) Radical Scavanging Assay:

DPPH is the most well-known and acceptable reagents used to hunt free radicals of plant extracts of both natural and synthetic origin (Marinova, 2011).

The antioxidant activity of different fractions and isolated compounds were determined in terms of hydrogen donating ability, using the DPPH method with a minor modification.

Principle (Marinova, 2011)

This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H. A freshly prepared DPPH solution exhibits a deep purple color. The transformation results in color change from purple to yellow, which is measured spectrophotometrically. Thus the antioxidant molecules will neutralize DPPH free radicals through converting them into colorless products (2, 2-diphenyl-1-hydrazine, or a substituted analogous hydrazine) resulting in a decrease absorbance.

Therefore, the potency of antioxidant activity is inversely proportional to the rate of decrease in wavelength. In this experiment, BHT (butyl-1- hydroxy toluene) and ASA (ascorbic acid) are used as positive control.

Experimental procedure:

At first, 2ml of methanol solution of the sample extracts and standard were prepared at various concentrations. Then, freshly prepared DPPH solution (3ml) were added to the test tubes. After that, the test tubes were kept for half an hour in darkened place for the accomplishment of the reaction. Lastly, at 517 nm wavelength absorbance of all the solutions were measured using a UV spectrophotometer.

$\% I = \{(A_o - A_1)/A_o\} \times 100$

where,

A_0 is the absorbance of the control, and

A_1 is the absorbance of the extract/standard

Then %I of inhibitionwere plotted against concentration(µg/ml) and from the graph IC_{50} value was calculated.

3.3 Brine Shrimp Lethality Bioassay

3.3.1 Principle (Olowa &Nuneza, 2013)

Brines shrimp bioassay is the most recognized and accepted method for determining cytotoxic effect of extract of any plant constitute. At first, in order to get nauplii, the brine shrimp eggs are hatched in simulated sea water. Then required amount of dimethylsulphoxide (DMSO) were added so that the desired concentration of the test samples can be prepared. When the hatching was done, the nauplii were counted by inspecting visually. They were taken into vials containing simulated sea water (5ml) and then the samples of different concentrations were added to the pre-labelled vials through micropipette. After that the vials were kept about for a day and survivors are counted after 24 hours.

3.3.2 Materials

Brine shrimp eggs (Artemiasalina leach)	Glass vials

Sea salt(NaCl)	Magnifying glass
Test samples of experimented plants	Lamp to attract shrimps
Small tank with perforated dividing damto hatch the shrimp	Test tubes
Micropipette	pipettes

Table 3.1: Test samples of experimental plant

Plant part	Sample code	Test sample	Calculated amount(mg)
Seeds of *Piper nigrum Linn*	ME	Methanolic extract partitionate	4.0
	CE	Chloroform extract partitionate	4.0
	NHE	n-hexane extract partitionate	4.0
	PEE	Petroleum ether extract partitionate	4.0

3.3.3 Experimental Procedure

Preparation of seawater

At first, 38g of sea salt (pure NaCl) was weighed and then dissolved in one litre of distilled water. After that the solution was filtered to get clear solution.

Hatching of brine shrimps

From nearby pet shops, the brine shrimp eggs of the test organism named *Artemiasalina* leach were collected and added to the small tank containing sea water. The rest portion of the tank was covered. Then the eggs were allowed to get hatched and matured into nauplii by one day. Throughout the hatching time, constant oxygen supply was provided to the tank. The tank was under the lamp through the perforated dam attracting the brine shrimp eggs

and they were taken for carrying out the experiment. About ten living shrimps were added to all of the test tubes containing different sample concentrations with the help of Pasteur pipette.

Counting of nauplii

After a day (24 hours) the number of survivors were counted through inspection of the vials by magnifying glass. For each diluted concentrations, the percentages of mortality were counted. Then the data were analyzed statistically using linear regression from a simple IBM-PC program. The effectiveness of the concentration-mortality relationship of plant product is usually expresses as median lethal concentration (LC_{50}) value. This indicates the concentration causing death in half of the test subjects after a fixed exposure period.

Chapter Four: Results And Discussion of Antioxidant Assay

4.1 Preparation of crude solvent extract

The sample plant part that has been used in this investigation is the ripe fruits' seeds of *Piper nigrum Linn* of Piperaceae family. At first, the seeds were sun dried for 3-5 days so that they can get rid of the moisture content. Then the dried seeds were grounded into coarse powder by using a high capacity grinding machine and were reserved in dark,cool and dry place for further use. The dried coarse powder (750gm) were extracted successively with methanol (500ml), chloroform(500ml) and n-hexane (500ml) solvents. After a week,all the extracts were filtered individually using filter papers. Then they (solvents) were evaporated separately at elevated temperature to dryness under reduced pressure in order to get dry extracts which yields to crude methanol extract (CME-8.06gm), crude n-hexane extract (CNE-1.65gm), crude chloroform extract (CCE-6.86gm).

Initially the samples were dried under sunlight for a period of 3-5 days to rid of the moisture content. The dried samples were made into a coarse powder using a grinding machine and stored in a cool,dark place. Samples (750gm) were then extracted separately 250gm for each with methanol (500ml), chloroform(500ml) and n-hexane (500ml) solvents. After seven days the extracts were separately filtered through filter paper and evaporated to a dry state under reduced pressure. This was done to make a dry extract that can be used to yield crude methanol extract (CME-8.06gm), crude n-hexane extract (CNE-1.65gm), crude chloroform extract (CCE-6.86gm)

Table 4.1 Different extracts obtained after extraction of *P. nigrum*

Name of the Partitionates	Weight of the partitionates (gm)
CME (Crude methanolic extract)	8.06
CCE (Crude chloroform extract)	6.86
CNE (Crude n-hexane extract)	1.65

4.2 Determination of total phenol content :

The main reagent used in this experiment is the Folin-Ciocalteu reagent. Phenolic content of crude methanolicextract(CME), crude chloroform extract(CCE) and crude n-hexane(CNE) extract of *Piper nigrum* were determined by using this mostly accepted reagent. The phenol content of these three fractions were calculated on the basis of the standard curve ofgallic acid which is shown in table 4.2 and figure 4.1. The results were expressed as the ratio of the mg concentration of gallicacidextract(GAE)/ gm of dried extracts. The average values were taken and expressed as mean± STD (Ahmed et al., 2014)

Table 4.2 Absorbance of gallic acid at different concentration treating with FCR reagent

Concentration (µg/ml)	Absorbance			Absorbance Mean ± STD
	a	b	C	
1	0.078	0.075	0.076	0.076 ± 0.001
2	0.176	0.171	0.181	0.176 ± 0.005
4	0.364	0.368	0.372	0.368 ± 0.004
8	0.722	0.718	0.726	0.722 ± 0.004
16	1.413	1.417	1.423	1.417 ± 0.005
32	2.758	2.752	2.764	2.758 ± 0.006

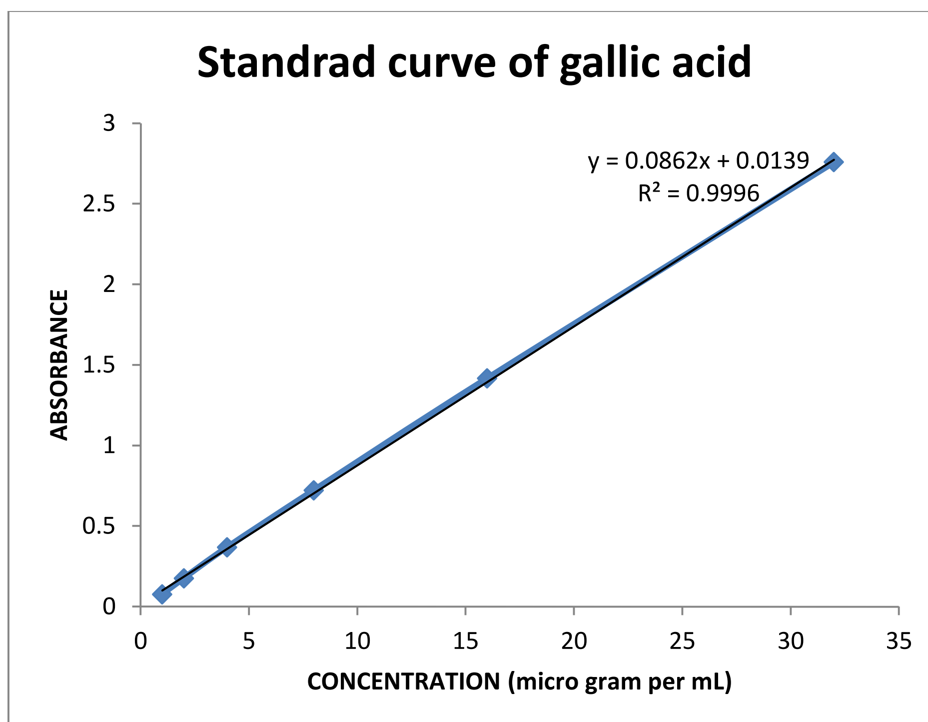


Fig: 4.1. Standard curve of gallic acid for the determination of total phenolic content.

The calculation for determining total phenol content of crude methanolic extract (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE) are shown in the table 4.3 below and figure 4.2

Table 4.3: Determination of total phenolic content of the crude methanolic extract (CME), crude chloroform extract (CCE) and crude n-hexaneextract(CNE) of *P. nigrum.*

Sample	No.of sample	Concentration (µg/ml)	Absorbance	GAE/gm of dried sample	GAE/gm of dried sample Mean ± STD
Crude methanolic Extract	1.	250	0.432	38.97	38.97±0.745
	2.	250	0.440	39.72	
	3.	250	0.424	38.23	
Crude	1.	250	0.574	52.184	52.86±0.745

chloroform extract	2.	250	0.576	52.376	
	3.	250	0.594	54.048	
Crude n-hexane extract	1.	250	0.466	42.136	40.50±1.198
	2.	250	0.446	40.282	
	3.	250	0.442	39.904	

The result of the total phenolic content showed that crude methanolic extract (CME),crude chloroform extract (CCE) and crude n-hexane extract (CNE) yielded 38.70±0.745,52.46±1.160 and 40.50± 1.198 GAE/gm of dried sample respectively. The result demonstrated that the total phenolic content of crude chloroform extract (CCE) is higher than that of crude methanolic extract (CME), and crude n-hexane extract (CNE).

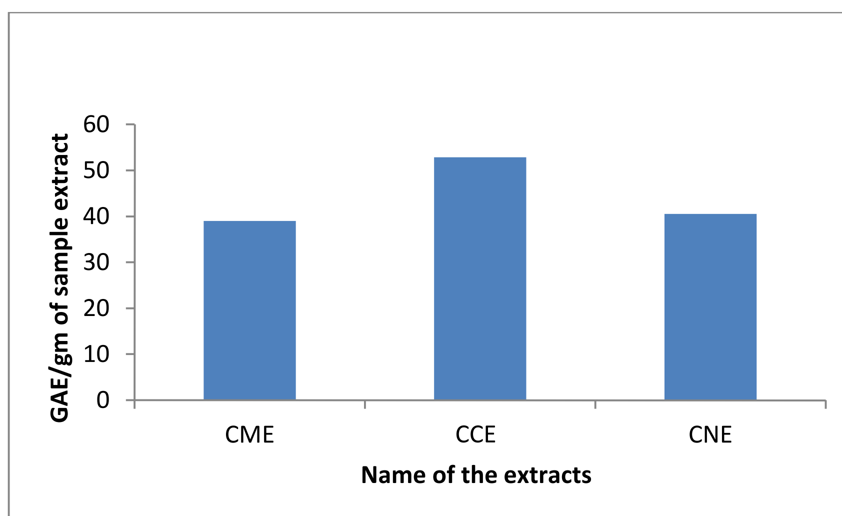


Fig. 4.2: Total phenolic content (µg/gm plant extract in gallic acid equivalent) of the crude methanolic extract (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE) of *Piper nigrum.*

4.3. Determination of total flavonoid content :

The total flavonoid content of crude methanolic extract (CME),crude chloroform extract

(CCE) and crude n-hexane extract (CNE) of *P. nigrum*are shown in table 4.4. and figure 4.3. The results were expressed as mg of catechin equivalent per gram of dried sample. The values represented the mean of triplicates ± STD of crude methanolic extract (CME),crude chloroform extract (CCE) and crude n-hexane extract (CNE). The outcomes were shown as mg of catechin equivalent/gm of dry sample. The values give the mean and standard deviation of the three partitionates of the extracts of P.nigrum (Kadam et al., 2013)

Table.4.4. Absorbance of catechin(standard) at different concentrations for quantitative determination of total flavonoids.

Concentration (µg/ml)	Absorbance			Absorbance
	a	b	c	Mean ±STD
31.25	0.241	0.225	0.260	0.242 ± 0.017
62.5	0.380	0.398	0.362	0.380 ±0.018
125	0.726	0.722	0.731	0.726 ± 0.004
250	1.476	1.481	1.468	1.475 ± 0.006
500	2.667	2.670	2.599	2.645 ± 0.040

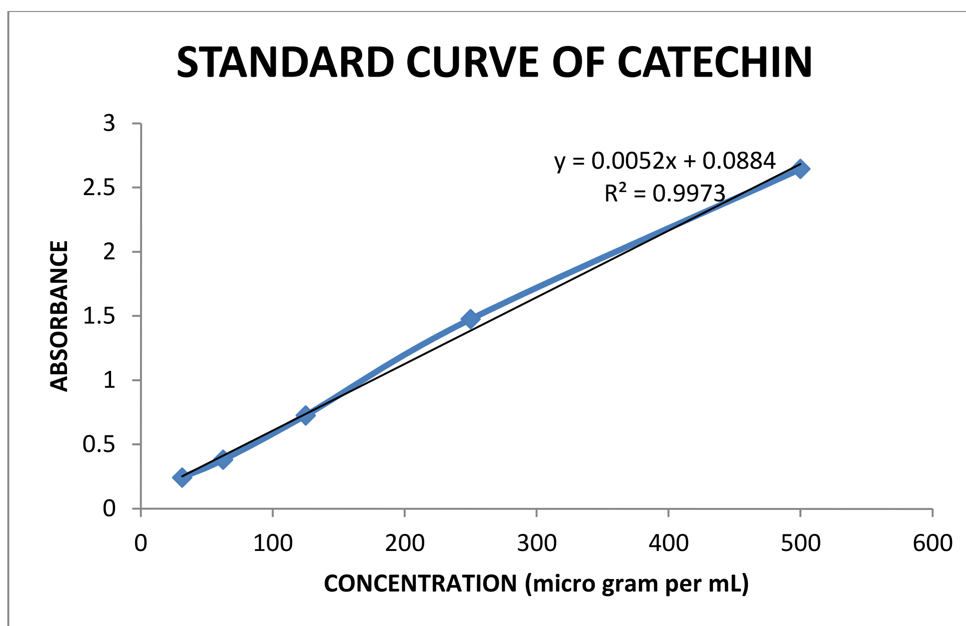


Fig. 4.3: Standard curve of catechin for the determination of total flavonoids.

The results (Table 4.5)showed that, total flavonoid content (TFC) of crude methanolic extract (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE) were 13.674±0.745, 39.564±1.420 and 32.56±1.198 µg of GAE/gm of dried extract respectively, while the total flavonoid content of crude chloroform extract (CCE) was 53.64±1.160 µg of catechin equivalent/ gm of dried extract. These findings demonstrated that the total flavonoid content of crude chloroform extract (CCE) was higher than that of crude methanolic extract (CME)**,** crude ethyl chloroform extract (CCE) and crude n-hexane extract (CNE) (Figure 4.4)

Table 4.5: Determination of total flavonoid content of the crude methanol extract (CME), crude chloroform extract (CCE)and crude n-haxane extract (CNE)of *P. nigrum.*

Sample	No.of sample	Concentration (µg/ml)	Absorbance	CAT/gm of dried sample	CAT/gm of dried sample Mean ± STD
Crude	1.	250	0.170	13.21	13.647±0.745

methanolic extract	2.	250	0.160	13.40	
	3.	250	0.158	14.33	
Crude chloroform extract	1.	250	0.590	53.40	53.64±1.160
	2.	250	0.584	52.84	
	3.	250	0.598	54.14	
Crude n-hexane extract	1.	250	0.372	33.116	32.56±1.198
	2.	250	0.378	30.882	
	3.	250	0.378	33.67	

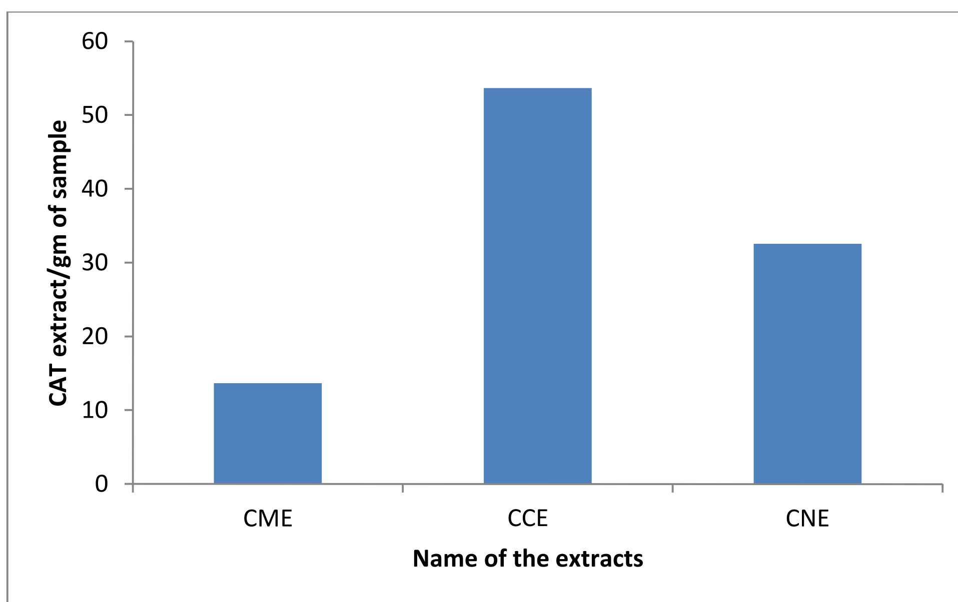


Fig: 4. 4. Total flavonoid content (µg /gm plant extract in catechin equivalent) of the crude methanolic extract (CME), crude chloroform extract (CCE) and crude n-hexaneextract(CNE) of *P. nigrum Linn.*

4.4. DPPH Radical Scavanging Activity

DPPH antioxidant assay is written on the capacity of 1,1diphenyl-2-picryl-hydrazyl, a steady free radical, to change color in the presence of if antioxidants are present. DPPH contains a

lose electron which gives it an absorbance at 517nm and is responsible for its dark purple appearance. Upon accepting an electron, DPPH changes color and this is measured from the change in absorbance and subsequently the percentage scavenging ability can be measured. This was catalyzed by increasing the concentration of the sample.The antioxidant activity of the crude methanolic extracts (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE) of *P. nigrum* were evaluated by DPPH radical scavenging assay.

In this investigation, the crude mathanolic extract (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE) were subjected for estimating the free radical scavenging activity. The IC_{50} value was found lowest in chloroform extract which means that it has the highest anti-oxidant activity whereas the IC_{50} value was highest in methanolic extract. The table 4.6 and figure 4.5 shows the different DPPH radical scavenging activity among all the three partitionates. Here, BHT (butyl-1-hydroxy toluene) and ASA (ascorbic acid) were used as standard against the partitionates (Marinova, 2011).

Table: 4.6. IC_{50} (µg/ml)values of crude methanol extract (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE) of *P. nigrum Linn* and BHT (Standard) for DPPH radical scavenging activity

Sample Code	Test Sample	IC_{50} value
BHT	Butyl-1- hydroxyl toluene	28.30
ASA	Ascorbic acid extract	5.90
CCE	Crude chloroform extract	58.98
CNE	Crude n-hexane extract	113.53
CME	Crude methanolic extract	168.75

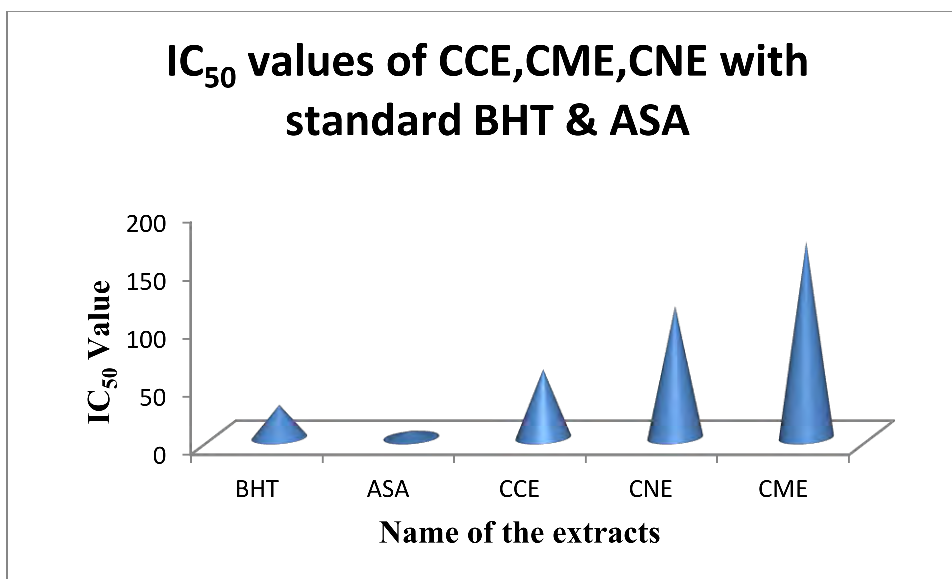


Fig: 4.5: IC_{50} (µg/ml)values of crude methanol extract (CME), crude chloroform extract (CCE) and crude n-hexane extract (CNE) of *P. nigrum Linn* andBHT,ASA (Standard) for DPPH radical scavenging activity.

Table 4.7: IC_{50} value of tert-butyl-1-hydroxy toluene (BHT)

Absorbance of the blank	Concentration (µg/ml)	Absorbance of the extract	% of inhibition	IC_{50} value µg/ml
0.324	0.977	0.287	11.42	28.30
	1.953	0.238	26.54	
	3.906	0.225	30.56	
	7.813	0.206	36.42	
	15.625	0.175	45.99	
	31.25	0.159	50.93	
	62.5	0.135	58.33	
	125	0.097	70.06	
	250	0.068	79.01	
	500	0.018	94.44	

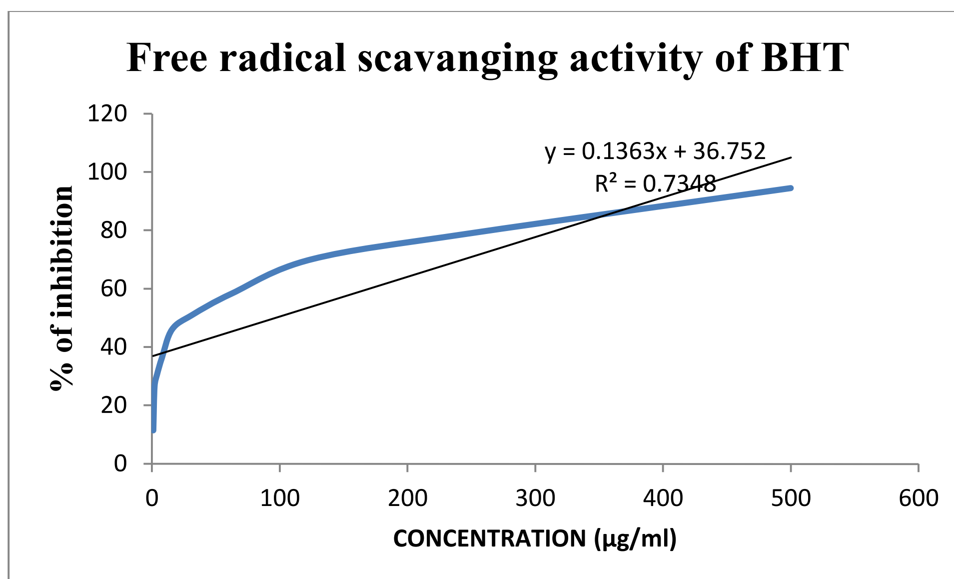


Figure 4.6: IC50 value of tert-butyl-1-hydroxy toluene (BHT)

Table 4.8: IC_{50} value of ascorbic acid (ASA)

Absorbance of the blank	Concentration (µg/ml)	Absorbance of the extract	% of inhibition	IC_{50} value µg/ml
0.324	0.977	0.193	40.43	5.90
	1.953	0.175	45.99	
	3.906	0.186	42.59	
	7.813	0.139	57.10	
	15.625	0.098	69.75	
	31.25	0.068	79.01	
	62.5	0.024	92.59	
	125	0.015	95.37	
	250	0.006	98.15	
	500	0.005	98.46	

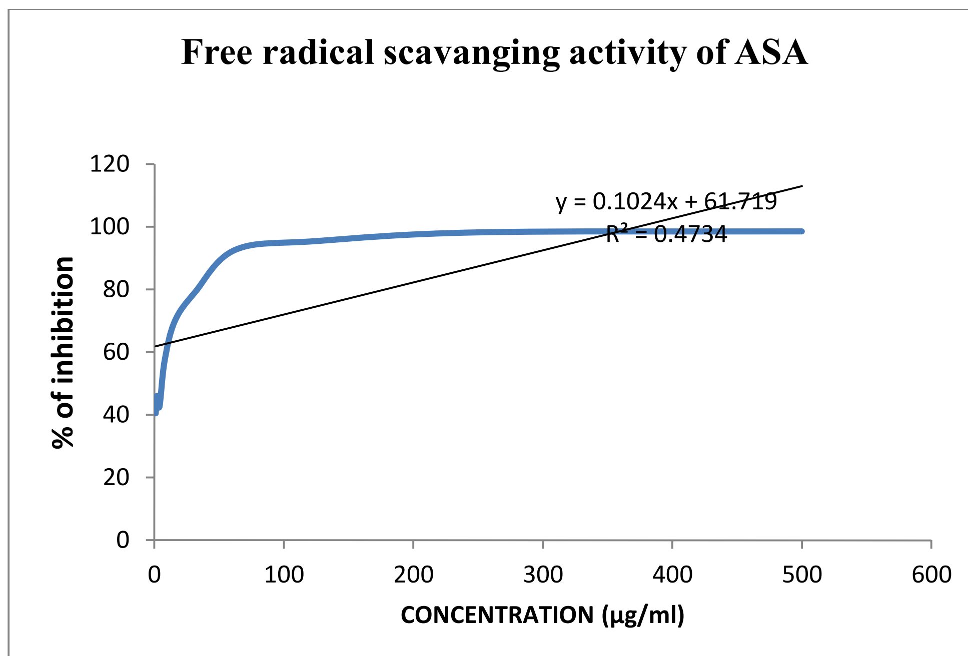


Figure4.7 : IC_{50} value of ascorbic acid (ASA)

Table 4.9: IC_{50} value of chloroform extract

Absorbance of the blank	Concentration (µg/ml)	% of inhibition	IC_{50} value µg/ml
0.324	1.953	0.26	58.98
	3.906	3.61	
	7.813	6.30	
	15.625	10.78	
	31.25	26.94	
	62.5	52.96	
	125	77.99	
	250	89.09	

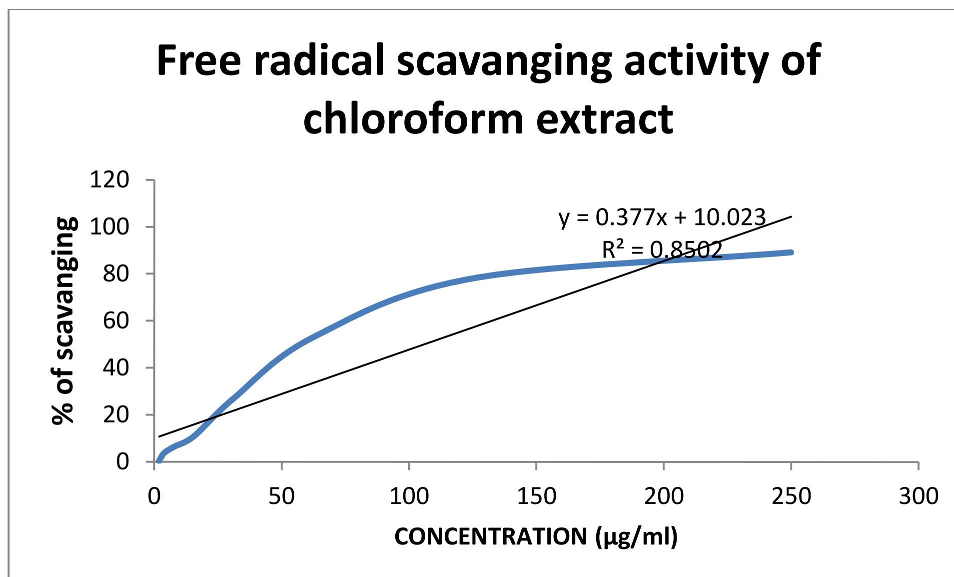


Figure 4.8: IC_{50} value of chloroform extract

Table4.10 : IC_{50} value of n-hexane extract

Absorbance of the blank	Concentration (µg/ml)	% of inhibition	IC_{50} value µg/ml
0.324	1.953	0.96	113.53
	3.906	3.27	
	7.813	5.64	
	15.625	7.84	
	31.25	14.85	
	62.5	32.84	
	125	53.85	
	250	66.92	

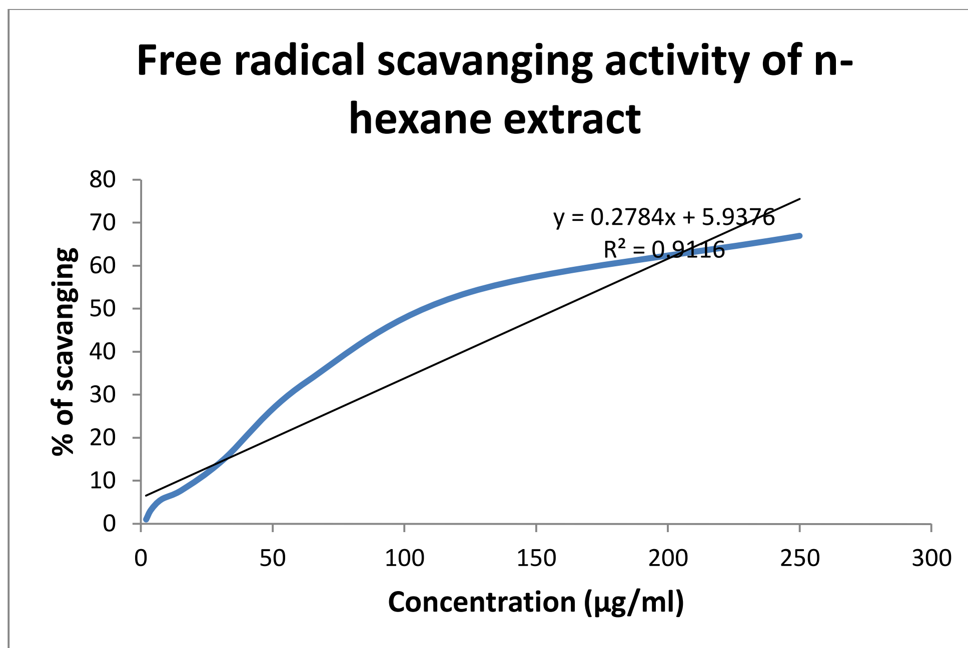


Figure 4.9: IC_{50} value of n-hexane extract

Table 4.11: IC_{50} value of methanol extract

Absorbance of the blank	Concentration (µg/ml)	% of inhibition	IC_{50} value µg/ml
0.324	1.953	0.76	168.75
	3.906	5.45	
	7.813	17.76	
	15.625	36.72	
	31.25	41.94	
	62.5	43.40	
	125	47.48	
	250	54.68	

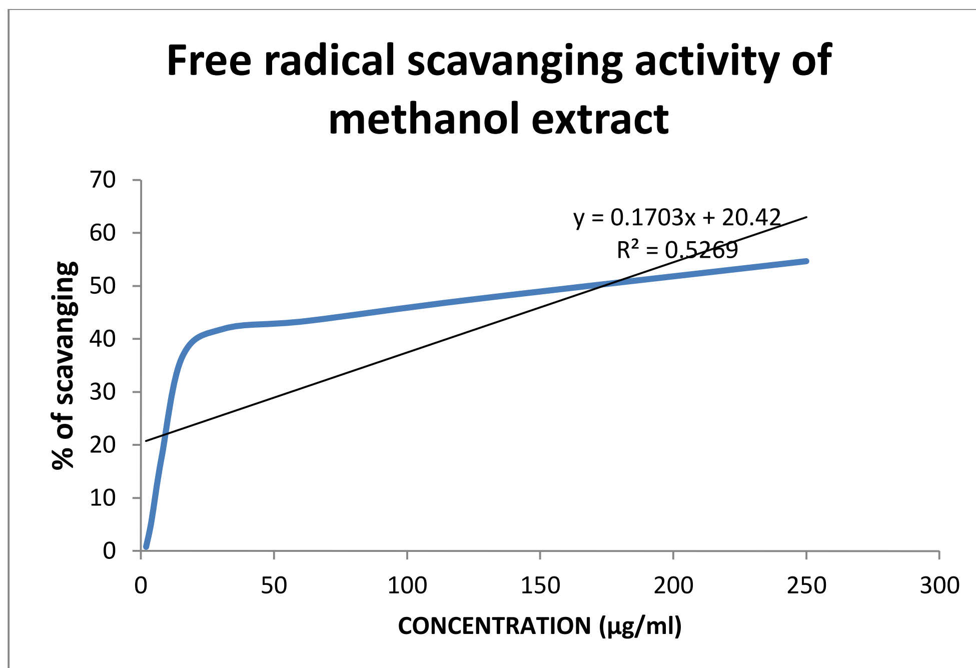


Figure 4.10: IC_{50} value of methanol extract

Chapter Five:
Results and Discussion of Brine Shrimp Lethality Bioassay

5.1. Preparation of test samples of the experimental plant

In order to prepare stock solution,each of the test samples (Table 5.1) are taken in vials and dissolved in 100 µL of pure DMSO (dimethyl sulphoxide). Then in the first test tube containing 5ml of simulated sea water, 50 µL of the solution was taken with 10 shrimp nauplii. So the final concentration of the first test tube becomes 400 µg/mL. After going through the serial dilution method, a series of solutions of varying concentrations were prepared from the stock solution. In each case, 50 µL of sample and 50 µL of DMSO were added to the vials. Therefore, different concentrations were found in all test tubes.

Table 5.1: Test samples with concentration values after serial dilution

Test tube number	Concentration (µg/ml)
1	400
2	200
3	100
4	50
5	25
6	12.5
7	6.25
8	3.125
9	1.5625
10	0.78125

5.2 Preparation of control group

In cytotoxicity study, control groups are used to validate the test methods which ensure that the results obtained are according to the activity of the test agents nullifying the effects of other possible factors. Two types of control groups are prepared which are-

- Positive control
- Negative control

5.3Preparation of the positive control group

In this study, vincristine sulphate was used as the positive control which is a widely accepted cytotoxic agent. The result of the test agents are compared with this one. To get an initial concentration of 20 µg/mL, calculated amount of vincristine sulphate was dissolved in DMSO. Then the serial dilution method is being implied in order to get concentrations of 10 µg/mL, 5 µg/mL, 2.5µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.3125 µg/mL, 0.15625 µg/mL, 0.078125 µg/mL, 0.0390 µg/mL. After that, the positive control solutions are added to the pre-marked vials containing ten living shrimp nauplii in 5ml simulated sea water.

5.4. Preparation of the negative control group

Three glass vials were pre-marked containing 5 mL of simulated sea water and 100 µL of DMSO was added to each of the vials with ten shrimp nauplii to be used as control groups. In these vials, if the brine shrimps show a rapid mortality rate then the experiment would be considered as invalid as most of the nauplii died because of some reasons other than the cytotoxicity of the compounds.

5.5 Counting of nauplii

After a day (24 hours) the number of survivors were counted through inspection of the vials by magnifying glass. For each diluted concentrations, the percentages of mortality were counted. Then the data were analyzed statistically using linear regression from a simple IBM-PC program. The effectiveness of the concentration-mortality relationship of plant product is usually expresses as median lethal concentration (LC_{50}) value. This indicates the concentration causing death in half of the test subjects after a fixed exposure period.

5.6 Result and discussion of the test samples of *P.nigrum*

The lethal concentration LC_{50} of the test samples were determined by plotting the percentage of mortality rate of the brine shrimps against the logarithm of the sample concentration and the curve data of regression analysis helps in obtaining the best fit-line. In the present study, vincristine sulphatewas used as positive control and the LC_{50} value was found to be 0.451µg/mL.The positive control VS gave significant mortality compared to the negative control. The LC_{50} values of diiferentpartitionate of extracts were compared to the positive control.

Table 5.2 : LC50 values of the test samples of *P.nigrum Linn*

Test samples	Regression Line	R^2	LC_{50} value (µg/ml)
VS (vincristine sulphate)	Y=30.79X+60.65	0.973	0.451
CE (chloroform extract)	Y=27.323X+26.388	0.8732	7.3282
ME (methanol extract)	Y=38.294X+15.818	0.9199	7.8163
NHE (n-hexane extract)	Y=23.156X+31.659	0.9278	10.1391
PEE (petroleum ether extract)	Y=23.009X+37.413	0.9656	3.524

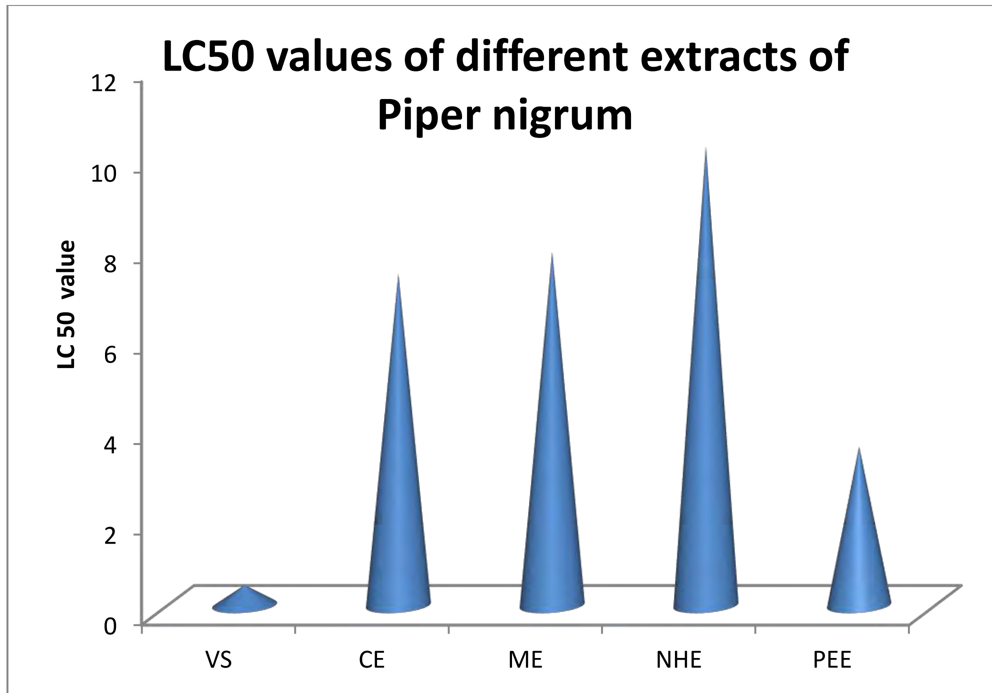


Figure 5.1 : LC_{50} values of the different extractives of *P.nigrum* Linn

Table 5.3: Effect of Vincristine sulphate(positive control) on shrimp nauplii

Concentration(micro gram per ml)	Log10 conc	% of mortalilty	LC_{50} value (μg/ml)
0	0	20	0.451
0.0390	-1.4089	20	
0.078125	-1.1072	30	
0.15625	-0.8061	30	
0.3125	-0.5051	40	
0.625	-0.2014	50	
1.25	0.09691	70	
2.5	0.39794	80	
5	0.6989	80	
10	1.00	90	
20	1.3010	100	

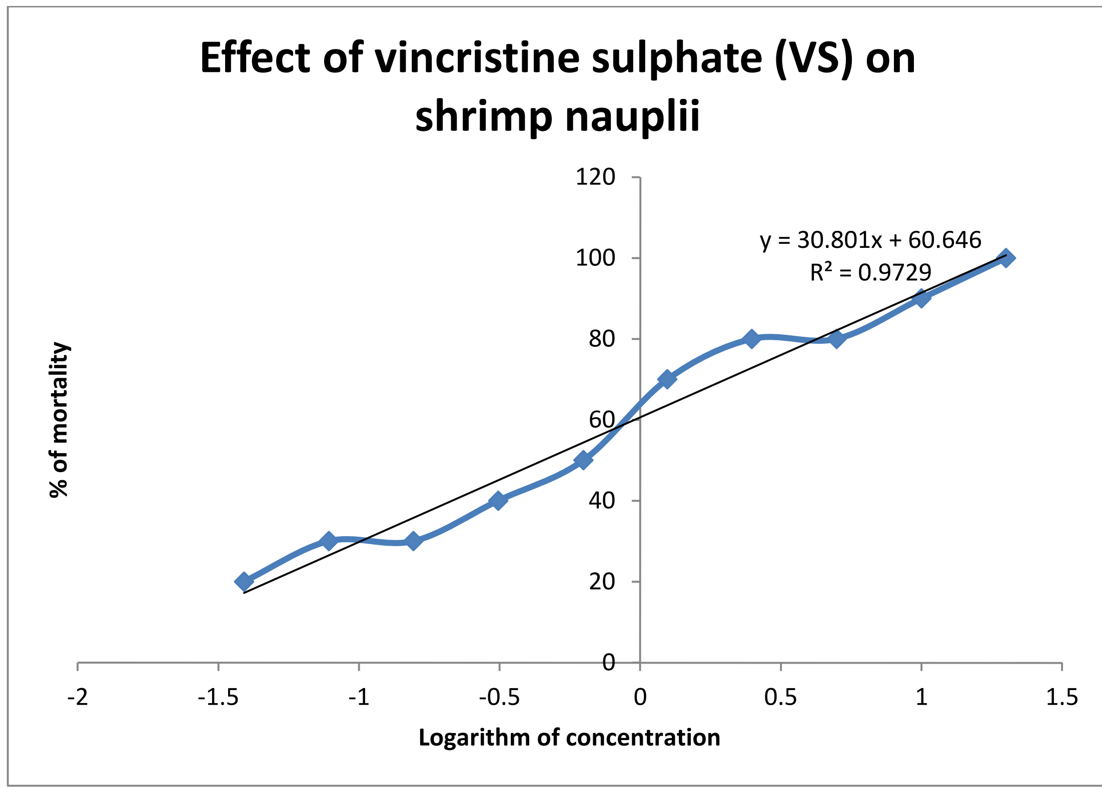


Figure 5.2 : Plot of % of mortality and predicted regression line of VS

Table 5.4 Effect of chloroform extract on shrimp nauplii

Concentration(micro gram per ml)	Log10 conc.	% of mortalilty	LC_{50} value (µg/ml)
0.78	-0.1079	10	7.3282
1.56	0.1931	22.5	
3.125	0.4948	44.4	
6.25	0.7958	66	
12.5	1.0969	66	
25	1.3979	70.5	
50	1.6989	70.5	
100	2	77.27	
200	2.301	87.5	
400	2.602	90	

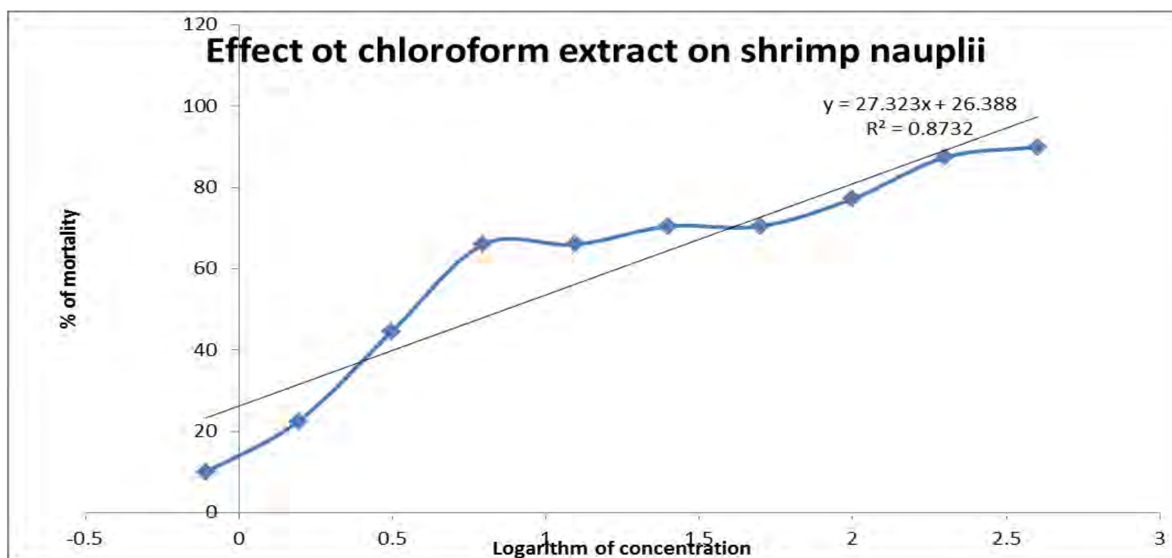


Figure 5.3: Plot of % Of mortality and predicted regression line of CE

Table 5.5 Effect of methanolic extract on shrimp nauplii

Concentratio(mico gram per ml)	Log10 conc.	% of mortalilty	LC_{50} value (µg/ml)
0.78	-0.1079	0	7.8163
1.56	0.1931	16.6	
3.125	0.4948	32.8	
6.25	0.7958	50	
12.5	1.0969	72.7	
25	1.3979	83.3	
50	1.6989	90	
100	2	90.4	
200	2.301	100	
400	2.602	100	

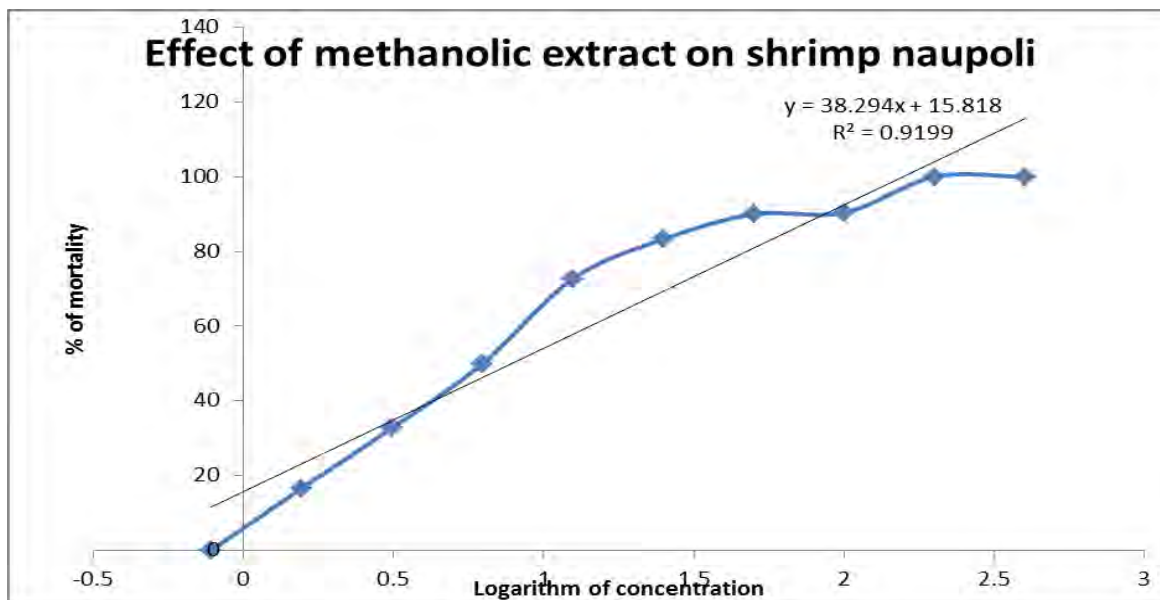


Figure 5.4: plot of % of mortality and predicted regression line of ME

Table 5.6: Effect of n-hexane extract on shrimp nauplii

Concentration(micro gram per ml)	Log10 conc.	% of mortalilty	LC_{50} value (µg/ml)
0.78	-0.1079	30.7	10.1391
1.56	0.1931	37.5	
3.125	0.4948	50	
6.25	0.7958	50	
12.5	1.0969	44.4	
25	1.3979	66.6	
50	1.6989	66.6	
100	2	75	
200	2.301	84.6	
400	2.602	100	

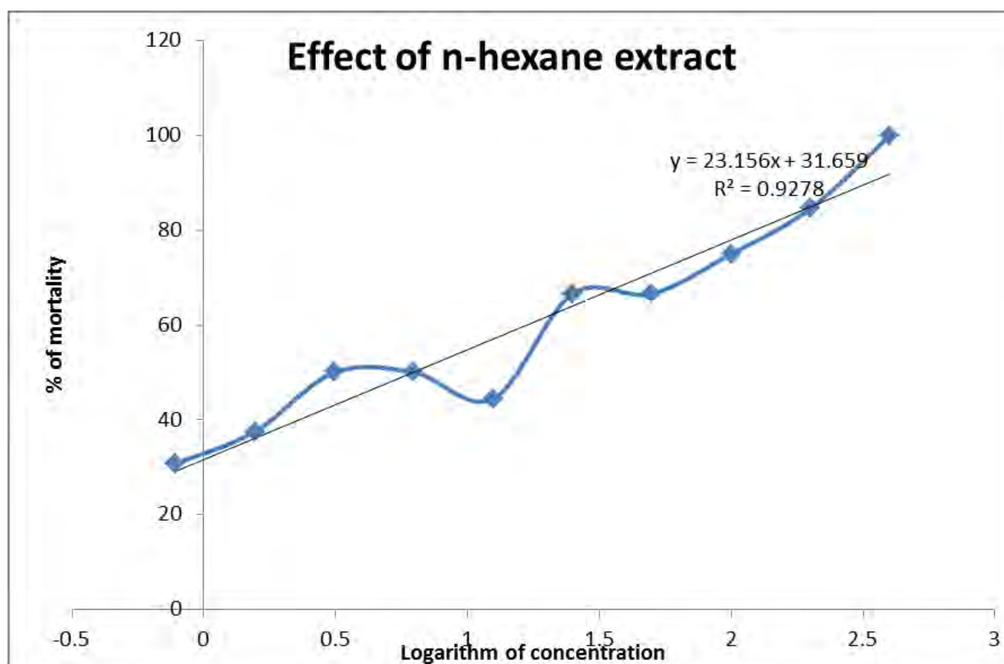


Figure 5.5: Plot of % of mortality and predicted regression line of NHE

Table 5.7Effect of petroleum ether extract on shrimp nauplii

Concentration(micro gram per ml)	Log10 conc.	% of mortalilty	LC_{50} value (µg/ml)
0.78	-0.1079	30	3.524
1.56	0.1931	50	
3.125	0.4948	50	
6.25	0.7958	54.5	
12.5	1.0969	60	
25	1.3979	66.6	
50	1.6989	80	

100	2	80	
200	2.301	90	
400	2.602	100	

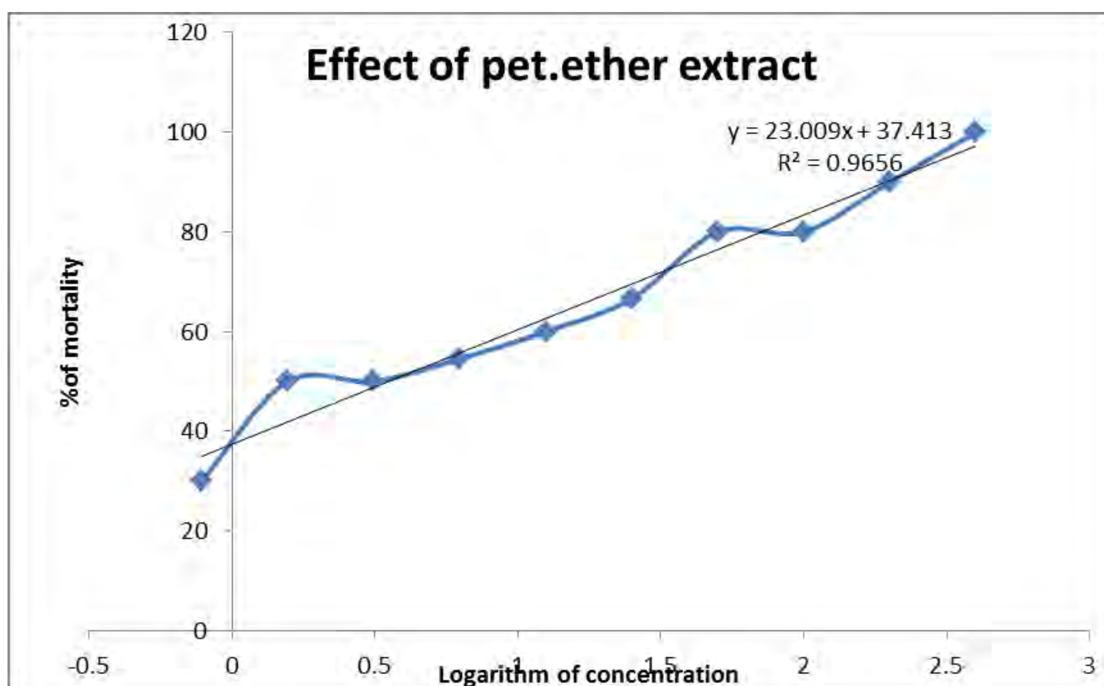


Figure 5.6: Plot of % of mortality and predicted regression line of PEE

The LC_{50} values of chloroform extract, methanolic extract, petroleum ether extract and n-hexane extract were found to be 7.3282µg/mL, 7.8163 µg/mL, 3.524µg/mL and 10.1391µg/mL respectively (Table 5.2) whereas the LC_{50} value of Vincristine sulphate and Ascorbic acid were found to be 0.451 µg/mL. So, the less the LC_{50} value, the less toxicity the compound will have. In this experiment, the LC_{50} value of petroleum ether extract was found to be lowest one among the four patitionates as can be inferred to possess moderate cytotoxic property.

Chapter Six: Conclusion

Crude extracts of white pepper could be a source to to procure new and effecient herbal medicine beside the synthetic antioxidants to treat oxidative stress related disorders. In this study, the different partitionates of *P.nigrum*(white pepper extract) were subjected for phytochemical and biological investigation.

Investigation involved the determination of two most important phytochemical contents which were the total phenol and flavonoid content among the three different fractions of white pepper extract. The results showed that, the chloroform extract of *P.nigrum*were found to have highest phenol (52.86±0.745 GAE/gmof dried sample)and flavonoid content (53.64±1.160 CAT/gm of dried sample) compared to the rest of the two partitionates.

Furthermore, biological investigation were done specially to determine the in-vitro antioxidant and cytotoxic activities of seed extracts of *P. nigrum* through DPPH test, to assess the free radical scavenging capacity, and brine shrimp assay respectively. IC_{50} value and LC_{50} value of different fractions of extracts were determined and compared with the control.

Therefore, this study clearly indicates that the extracts of *P.nigrum* may be a very important contributor in various drug discoveries especially as a natural source of potential antioxidant with less cytotoxic effect. However in vivo pharmacological evaluation needs to be done to validate the traditional use and to explore any other therapeutic activity along with their side effects to gather substantive understanding about the safety and efficacy of white pepper.Moreover, phytochemical analysis of the extract will be helpful for isolation and characterization of its active compounds.

References

Ahmed, A., Husain, A., Mujeeb, M., Khan, S. A., Aldrami, H. A. B. & Bhandari, A. (2015). Quantification of total phenol, flavonoid content and pharmacognostical evaluation including HPTLC fingerprinting for the standardization of *Piper nigrum Linn* fruits. *Asian Pacific Journal of Tropical Biomedicine*, 5(2), 101-107.

Agbora, G., Vinson, J. A., Oben, J. E. & Ngogang, J. Y. (2014). Comparative analysis of the in vitro antioxidant activity of white and black pepper. *Nutrition Research*, 2, 47. doi: 10.1016/j.nutres.2006.09.019

BD News24. (15th December, 2015). Cancer rates rising in lower-income countries: Study. Retrieved from http://bdnews24.com/health/2015/12/15/cancer-rates-rising-in-lower-income-countries-study

Fontenot, B. (2011). Antioxidants Explained :Why these coumpounds are so important, *The Atlantic Monthly Group*. Retrievedfromhttp://www.theatlantic.com/health/archive/2011/10/antioxidants-explained-why-these- compounds-are-so-important/247311/

Hasslberger, B. (2007). Networking for a better future: Synthetic antioxidants can harm your health. *Organic consumer association.* Retrieved fromhttps://www.organicconsumers.org/scientific/synthetic-antioxidants-can-harm-your-health

Irene, L. (2013). Piperaceae- the Pepper family. *Tropical Biodiversity*. Retrieved from http://blogs.reading.ac.uk/tropical-biodiversity/2013/04/piperaceae-the-pepper-family/

Kato, M. J. & Furlan, M. (2007). Chemistry and evolution of the Piperaceae. *IUPAC, Pure and Applied Chemistry*, 79(4), 529-538.doi: 10.1351/pac200779040529

Kadam, P. V., Yadav, F. A., Patel F. A., & Karjikar, M. J. (2013). Pharmacognostic, phytochemistry and physicochemical studies of *Piper nigrum Linn* fruit (Pipereaceae). *Int. Res. J. Pharm.*, 4(5), 189-193. doi: 10.7897/2230-8407.04538

Marinova, G. & Batchvarov, V. (2011). Evaluation of the methods for determination of the free radical scavenging activity by DPPH. *Bulg. J. Agric. Sci.,* 17, 11-24

Meghwal, M., & Goswami, (2012). Nutritional Constituent of Black Pepper as Medicinal Molecules: A Review. *Open Access Scientific Reports*, 1(1), 1-7. doi: 10.4172/ scientificreports. 129

Olowa, L. F. & Nuneza, O. M. (2013). Brine Shrimp Lethality Assay of the Ethanolic Extracts of Three Selected Species of Medicinal Plants from IliganCity, Philippines. *International Research Journal of Biological Science,* 2(11), 74-77. ISNN: 2278-3202

The Authors of Encyclopoedia,(2016). Piperaceae. *In Encyclopædia Britannica*. Retrieved from http://www.britannica.com/plant/Piperaceae

Purseglove, J. W. (1981). Handbook of herbs and spices. *Spices*, NY: Woodhead Publisher

References

Pisutthanan, S., Plianbangchang, P., Pisutthanan, N., Ruanruay, S. & Muanrit, O. (2004). Brine Shrimp Lethality Activity of Thai Medicinal Plants in the Family Meliaceae. *Naresuan University Journal*, 12(2), 13-18.

Shekhar, T. C. & Anju, G. (2014). Antioxidant Activity by DPPH Radical Scavenging Method of Ageratum conyzoides *Linn.* Leaves. *American Journal of Ethnomedicine*, 1(4), 244-249. ISNN: 2348-9502

Sidarta, Y. O., Prasetey, N., Fitriani, D. & Prawiro, S. R. (2013). White Pepper Extract (Piper nigrum L.) as Antibacterial Agent for Streptococcus mutans In Vitro. *IOSR-JDMS*, 4, 25-29.

Shanmugpriya, K., Saravana, P. S., Mohammed, P., & Williams, B. (2012). Antioxidant potential of pepper leaves and its antimicrobial potential against some pathogenic microbes,*Indian Journal of Natural Products and Resources*, 3(4), 570-577.

Singh, S., Kapoor, I. P. S., Singh, G., Schuff, C., Lamposona, M. P. & Catalan, C. A. N. (2013).Chemistry, Antioxidant and Antimicrobial Potentials of White Pepper (*Piper nigrum L.*) Essential Oil and Oleoresins. *Proceedings of the National Academy of Sciences*, 83(3), 357-366. doi: 10.1007/s40011-012-0148-4

Wang, X. & Quinn, P. J. (1999).Vitamin E and Its Function in Membranes. *NCBI*, 38(4), 309-336.

Yuncker, T. G. (1958). The Piperaceae- A Family Profile.*Brittonica*, 10(1), 1-7. doi: 10.2307/2804687