ANTIBACTERIAL, ANTIOXIDANT AND PLATELET BOOSTING PROPERTIES OF SEMALU AND MANNA PLUS EXTRACTS

by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
IC_{50}	Concentration of a test substance required for 50 % inhibition *in vitro*
$^{\bullet}OH$	Hydroxyl radical
°C	Degree Celsius
µ	Micro
µL	Micro litre
µmol GAE/g	Micromole gallic acid equivalent
µmol Tr/g	Micromole trolox equivalent
$AlCl_3$	Aluminum chloride
AMX	Amoxicillin
ANOVA	Analysis of variance
AR	Analytical grade
ATCC	American type culture collection
B. subtilis	*Bacillus subtilis*
CHCl3	Chloroform

Cm	Centimeter
CO_2	Carbon dioxide
CY	Cyclophosphamide
DMEM	Dulbecco`s modified eagle medium
DMSO	Dimethyl sulfoxide
DPPH	1, 1-diphenyl-2-picryl-hydrazyl
E. coli	*Escherichia coli*
ESI	Electron spray ionisation
FBS	Fetal bovine serum
FCR	Folin-Ciocalteu reagent
FDA	Food and drug administration, United states
$Fe(SO_4)_2$	Ferric sulphate
Fe^{3+}-TPTZ	Ferric tripyridyltriazine
Fe^{2+}-TPTZ	Ferrous tripyridyltriazine
$FeCl_3$	Ferric chloride
FRAP	Ferric reducing antioxidant power
G	Gram
GAE/g	Gallic acid per gram

HAT	Hydrogen atom transfer
HPTLC	High Performance thin layer Chromatography
Hrs	Hours
i.p	Intraperitoneal injection
INT	*para* iodonitrotetrazolium
MeOH	Methanol
mg/kg	Milligrams per kilogram
mg/mL	Milligram per millilitre
mg/mL	Milligrams per millilitre
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
Mins	Minutes
mL	Millilitre
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate
P. Aeruginosa	*Pseudomonas. Aeruginosa*

p.o	Post oral
PBS	Phosphate buffer saline
Ppm	Parts per million
ROOH	Hydro peroxides
ROS	Reactive oxygen species
Rpm	Revolution per minute
S. aureus	*Staphylococcus aureus*
S.E.M	Standard error mean
SD	Standard deviation
TLC	Thin layer chromatography
TE/g	Trolox per gram
U.K	United Kingdom
USA	United States of America
UTI	Urinary tract infection
UV	Ultra violet
v/v	Volume/volume
Vis	Visible
WB	Water bath

WHO	World Health Organization

ANTIBAKTERIA, ANTIOKSIDAN DAN AKTIVITI PENINGKATAN PARAS PLATLET EKSTRAK SEMALU DAN MANNA PLUS

ABSTRAK

Semalu dan Manna Plus adalah produk komersial yang terkenal dengan nilai-nilai perubatannya seperti meningkatkan imuniti, antibiotik semula jadi yang sederhana potensinya, metabolisme protein dan mengimbangkan homeostasis. Para penyokong atau pengguna produk-produk ini mendakwa bahawa ia berkesan dalam meningkatkan platlet pesakit denggi. Kajian cytotoxicity, antioksidan, antibakteria dan peningkatan kadar platelet dijalankan untuk menilai sifat-sifat farmakologi ekstrak semalu dan manna plus. Kaedah HPTLC telah berjaya diwujudkan untuk menganalisis fitokimia eugenol, kurkumin, asid linoleik dan kuercetin untuk tujuan kuantifikasi dan kawalan mutu. Ekstrak terpiawai semalu dan manna plus telah digunakan untuk semua penilaian farmakologi. Keputusan sitotoksin manna plus, ekstrak metanol dan ethanol semalu menunjukkan tiada kesan toksik pada kepekatan <620 μg / mL dalam sel Chang HeLa. Merujuk kepada aktiviti antimikrob, semalu dan manna plus ekstrak etanol didapati aktif terhadap *S. aureus, B. subtilis* dan *P. aeruginosa* (MIC: 125-250 μg/mL) manakala ekstrak air menunjukkan perencatan terhadap bakteria gram positif sahaja (MIC: 250 μg / mL). Ekstrak etanol dan metanol Semalu dan manna plus menunjukkan aktiviti antioksidan (DPPH: TE / g: 452,8 ± 1,1 -126,6 ± 2.0 mg; TPC: GAE / g: 153.7 ± 0,05-41,5 ± 0.02 mg; FRAP: TE / g: 192,49 ± 3,1-20,30 ± 2.0 mg). Kajian tikus yang teraruh trombositopenin, ekstrak etanol menunjukkan kesan peningkatan platelet setelah pemberian. Kesan peningkatan platelet ekstrak ini (5 mg/kg) didapati setara dengan ekstrak betik (500 mg/kg). Secara ringkasnya, ciri-ciri antioksidan, antibakteria dan sifat peningkatan

platelet produk semalu dan manna plus mungkin dapat memberikan sesetengah bukti saintifik bagi penggunaanya sebagai produk tambahan untuk merawat pelbagai penyakit termasuk denggi.

ANTIBACTERIAL, ANTIOXIDANT AND PLATELET BOOSTING PROPERTIES OF SEMALU AND MANNA PLUS EXTRACTS

ABSTRACT

Semalu and Manna Plus are commercial products known for their medicinal values such as boosting immunity, natural mild antibiotic, protein metabolism and balancing homeostasis. The proponents or consumers of these products claim that they are efficient in boosting platelets in dengue acquired patients. In view of this some pharmacological properties of these extracts such as, cytotoxicity, antioxidant, antibacterial and platelet boosting effect were undertaken. HPTLC methods were successfully developed for phytochemical analysis of eugenol, curcumin, linoleic acid and quercetin respectively for quantification and quality control purposes. Standardized extracts of semalu and manna plus were employed for all pharmacological evaluations. The cytotoxicity results demonstrated manna plus and semalu methanolic and ethanolic extracts exhibited nontoxic effects at <620 µg/mL on Chang HeLa cells. With regards to antimicrobial activity, semalu and manna plus ethanolic extracts were active against *S. aureus, B. subtilis* and *P. aeruginosa* (MIC: 125-250 µg/mL). However water extracts showed inhibition only against gram positive (MIC: 250 µg/mL) bacteria. Semalu and manna plus ethanolic and methanolic extracts demonstrated antioxidant activity (DPPH: TE/g: 452.8 ± 1.1 - 126.6 ± 2.0 mg; TPC: GAE/g: 153.7 ± 0.05 – 41.5 ± 0.02 mg; FRAP: TE/g: 192.49 ± 3.1 - 20.30 ± 2.0 mg). With reference to platelet boosting properties ethanolic extracts demonstrated platelet boosting effects in thrombocytopenia induced rats. The platelet boosting effects of these extracts (5 mg/kg) are comparable to that of papaya extract (500 mg/kg). In summary, the antioxidant, antibacterial and platelet

boosting properties of semalu and manna plus may provide some scientific evidence for its usage as supplement for various ailment including dengue.

CHAPTER 1

INTRODUCTION

There are numerous commercial herbal mixtures available in the market around the world that is often sold as supplement product for promotion of general health. In crude form or extracts are freely available everywhere including at supermarkets, discount stores, and convenience stores. They are not regulated like allopathic drugs which must be tested rigorously before being allowed for human/patient consumption, unless if these supplements claim to prevent, treat or cure any specific disease.

Few such examples are herbal preparations of the plant *Echinacea purpurea*, which have immune-enhancing properties and are also reported to have positive effects in treating common flu (O'Neil *et al*., 2008). Milk thistle extracts have been used as a "liver tonic" for centuries. In recent years, silibinin, the active ingredient in milk thistle extracts, has been studied both in vitro and in vivo to evaluate the beneficial effects in hepatic disease. Silibinin increases antioxidant concentrations and improves outcomes in hepatic diseases resulting from oxidant injury (Hackett *et al*., 2013; Freedman, *et al*., 2001; Vaknin *et al*., 2008). *Hypericum perforatum L*, also known St. John's wort is an important phytopharmaceutical, for treatment of mild to-moderately severe depression, has significantly increased in the last few years. One of the major active compound Hyperforin (a prenylated phloroglucinol) is utilized as the key compound in measuring the quality of St. Johns wort and is typically used as the measure of extract potency and studies suggest that it could be the antidepressive agent in St. John's wort. However, St. John's wort is one of the top-selling herbal products (Barnes *et al*., 2001; Butterweck, 2003).

In general these herbal products are designated as supplement to promote good health; but, some of these products are claimed to have medicinal properties for certain diseases. However such off-label claims are made by the consumers or the proponents without any reliable clinical evidence. This warrants a more objective evaluation to ascertain such claims. In recent years, Mas Ayu Corporation, a Malaysian base herbal product company has made such a claim on certain product for treatment of dengue. Semalu and Manna Plus are the products of this company, which are being sold as food supplement for general health and currently registered under food and beverage (Food Regulations Act, 1985). These two products are available in Malaysian market. They are a mixture of various spices, edible vegetables and herbal plants. The consumers and proponents of these products claim that the combine use of both products act as good supplement or adjunct for dengue treatment; as it is known to boost platelet counts and immunity when consumed by dengue patients (Asha Ravindran, 2013).

Though all the above pharmacological claims warrant in depth scientific investigation; but these products are perpetually being sold for their valuable medicinal properties besides its major claim for general health. This was mainly attributed to their ingredients (turmeric, ginger, garlic, clove, neem, jeera, etc.) which are known for their medicinal properties.

Interestingly, over the past years there are numerous claim by the proponents and consumers that both semalu and manna plus have been informally used in treatment of dengue patients. It is suggested to boost up platelet in patients with serious decline in platelets (Asha Ravindran, 2013). Severe thrombocytopenia and increased vascular permeability are two major characteristics of dengue hemorrhagic fever. The commonly recommended dose regimens are two capsules of

semalu and manna plus three times daily for a period of a week or two. In most cases, the subject continues this supplement for promotion of general health. However this claim warrants a more objective evaluation in terms of its pharmacological properties such as antioxidant, anti-thrombocytopenia, antiviral and cytotoxicity.

As there are two different types of dengue caused by the dengue virus namely dengue fever (not life threating and self-limited) and dengue haemorrhagic fever or dengue shock syndrome (life threatening leading to death) (Kao-Jean *et al*., 2000). Current treatment regimens are symptom based; there is no specific antiviral treatment currently available for dengue fever. Supportive care with analgesics, fluid replacement, and bed rest is usually sufficient. In severe cases of dengue haemorrhagic fever plasma leakage occurs, administration of intravenous fluids is necessary to maintain haematocrit levels. If plasma leakage reaches very low levels (i.e. >20000 x 10^9/L) the only treatment is blood transfusion (WHO, 1997).

However the above said anonymous medicinal claims of semalu and manna plus merits scientific investigation. In view of this, research was undertaken to determine some pharmacological properties of these products. Cytotoxicity testing is an integral part of any toxicity screening program of natural resources for medicinal properties. In this regard, work was carried out to first establish semalu and manna plus cytotoxicity using Chang HeLa liver cells. One of the medicinal claim of semalu and manna plus is their immunity boosting property; this justification was used by the proponents to boost platelet count in treating dengue patients. Platelets are the secondary line of defence within an immune response. With this in view, the platelet boosting properties of these products was investigated in thrombocytopenia rats. Several reports suggest that high levels of antioxidant within the humans

eliminate free radicals by decreasing oxidative stress that helps boost the immune system. Later study was conducted to determine their respective antioxidant properties. In addition to this, antibacterial activities of semalu and manna plus were also determined. The following objectives were undertaken in order to achieve the above mentioned goal.

1.1 Problem statement

How will the ethanolic extracts of Semalu and Manna Plus aid in the boosting of platelets, which is vital for recovery from thrombocytopenia and dengue fever and dengue shock syndrome.

1.2 Objectives

- To evaluate the blood platelet boosting properties of Semalu and Manna plus extracts as individual and in combination in healthy rats, as well as in thrombocytopenia-induced rats.
- To evaluate the extracts for *in vitro* cytotoxicity using HeLa Chang cell line.
- Fingerprint of Semalu and Manna plus extracts were obtained using HPTLC technique.
- To determine the antioxidant potential of Semalu and Manna Plus extracts.
- To evaluate the extracts for *in vitro* antibacterial activity.
- To prepare aqueous, ethanolic and methanolic extracts of Manna Plus and Semalu by maceration technique.

1.3 Hypotheses

If thrombocytopenia induced rats are treated with ethanolic extracts of Semalu and Manna Plus then platelet boosting effects in thrombocytopenia induced rats can be evaluated and proved.

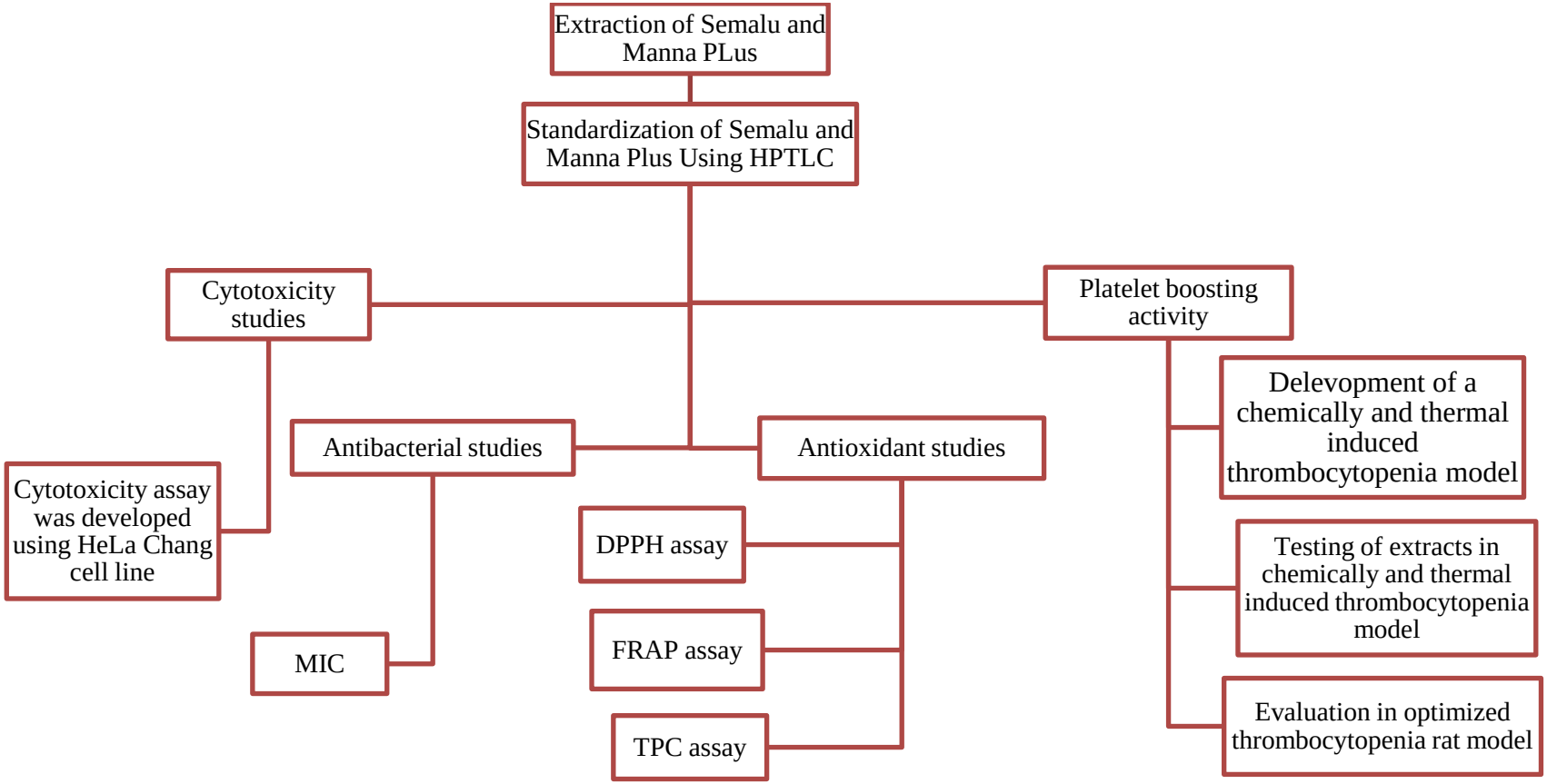

Figure 1.1: Flowchart of Research

CHAPTER 2

LITERATURE REVIEW

2.1 Semalu and Manna Plus

There are numerous commercial herbal mixtures available in the market around the world and are often sold as a supplement products that can promote general health. Herbal in crude form or extracts are freely available everywhere including at supermarkets, discount stores, and convenience stores. They are not regulated like allopathic drugs which must be tested rigorously before being allowed for human consumption, unless if these supplements claim to prevent, treat or cure any specific disease. In general these herbal products are designated as supplement to promote good health; but some of these products are claimed to have medicinal properties for certain diseases. However such off-label claims are made by the consumers or the proponents without any reliable clinical evidence.

This warrants a more objective evaluation to ascertain such claims. In recent years, Mas Ayu Corporation, a Malaysian base herbal product company has made such a claim on certain products for treatment of dengue. Semalu and Manna Plus are the products of this company, which are being sold as food supplement for general health and currently registered under food and beverage (Food Regulations Act, 1985). These two products are available in Malaysian market. They are a mixture of various spices, edible vegetables and herbal plants. The consumers and proponents of these products claim that the combine use of both products act as good supplement or adjunct for dengue treatment as it is seen to increase platelet counts and boost the immunity when consumed by dengue patients (Asha Ravindran, 2013).

2.1.1 Chemical constituents

Semalu is a mixture of spices, vegetables and one herb. It contains *Curcuma longa* radix (turmeric), *Allium sativium* bulbus (garlic), *Allium cepa* bulbus (onion) and *Azadirachta indica* follium (neem tree, herb). Manna plus contains *Allium sativium* bulbus (garlic), *Allium cepa* bulbus (onion), *Nigella sativa* semen (black cumin), *Cuminum cyminum* semen (cumin seeds), *Zingiber officinale* radix (ginger), *Syzygium aromaticum* flos (clove), *Coffea arabica* semen (coffee) & *Anethum graveolens* semen (dill). They are mostly an assortment of culinary spices. A spice is a dried seed, fruit, root, bark, or vegetable substance primarily used for flavouring, colouring or preserving food. Many spices have antimicrobial properties. This may explain why spices are more commonly used in warmer climates, which have more infectious disease. Semalu contains Neem a medicinal herb but in a very small proportion in the mixture. Manna contains coffee which is used as a brewed beverage and the most popular drink in the world. Onion, garlic, ginger are edible vegetables, which are used extensively in food preparation. Black cumin, cumin seeds, turmeric and clove are being used in the cuisines of many different cultures, in both whole and ground form. Onion, garlic, ginger are edible vegetables, which are used extensively in food preparation. Black cumin, cumin seeds, turmeric and clove are being used in the cuisines of many different cultures, in both whole and ground form.

2.2 Issues related to the herbal extracts preparation

2.2.1 Qualitative and quantitative measurements of herbal extracts

2.2.1.(a) **Standardisation**

Qualitative analysis is a vital process in determining the constituents present in crude herbal extracts and is a reliable part of quality control protocols as any change in the quality of the extract directly affects its constituents. Identification of the constituents in crude extracts is elementary necessity (Seasotiya *et al*., 2014). Variation and complexity of phytoconstituents present in herbal extracts makes the process of standardisation and quality control complex.

Several variations are outlined within the same plant material or different parts of that plant. These variations can be attributed to the geographic location, soil fertility and handling of these plants during harvesting, also age during harvesting. Multiple phytoconstituents present in herbal extracts that include active, inactive and unknown constituents that may contribute to their dietary importance but not therapeutics (Gupta, 2003). Therefore, methodologies that can promote developing a fingerprint of crude herbal extracts would be convenient to detect quality as well as stability of the extracts over time. Developmental methods should be derived on the basis of easy retrieval, reproducibility and electronic storage data analysis (Rehana & Nagarajan, 2014). Research on different extraction protocols and analytical method such as spectrophotometry, high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC–MS) and Fluorescence Transmission-Infrared Spectroscopy (FT-IR) are usually employed for the quality measurement of plant active/major compounds (Kirtikar & Basu, 2005).

2.2.1.(b) **Standardisation using HPTLC**

Methods based on high-performance thin layer chromatography (HPTLC) perhaps are regarded as a suitable replacement for TLC, as they are being studied as an imperative device in practise for drug analysis. Major advantage of HPTLC is its proficiency to evaluate numerous samples concurrently using minimal amounts of mobile phase that reduces time and cost of analysis. Additionally it minimises exposure risks and considerably lessens dumping of toxic organic effluents decreasing prospects environment pollution. HPTLC also enables repetitive detection of chromatogram with similar or different parameters (Sushma *et al*., 2013). Therefore the contemporary methods defining the identification and quantification of active constituents within an herbal drug could prove beneficial for suitable standardization of herbal drugs, extracts and its formulations. Likewise, WHO has emphasized the necessity to warrant the quality of the herbal plant products with medicinal values by means of modern controlled techniques and applying appropriate standards (Ranjit, 1992; WHO, 1989). HPTLC recommends better resolution and estimation of active constituents and can be performed with reasonable accuracy in a shorter period (Sethi, 1996).

2.2.2 Safety of herbal extracts

2.2.2.(a) Cytotoxicity

The attribute of being toxic to cells are called cytotoxicity. There are many toxic agents such as synthetic chemical substances and phytochemical substances. Necrosis (accidental cell death) or apoptosis (programmed cell death) can be induced to healthy living cells by using a cytotoxic compound. A cytotoxic agent or drug is toxic to laboratory cultured tumours whereas, drugs that are toxic to tumour cells in human clinical trials are referred as an anticancer agent. Therefore, cytotoxic studies are done as precursors in developing potential anticancer agents.

Humans have acquired great benefits from medicinal plants since ancient times. A variety of these plants are traditionally used to treat cancers in the Asian cultures (Lee *et al*., 2007). Discovery of new, impregnable and efficacious drugs are subjected to the importance of pharmacological screening of plants. Statistically around 80% of humans use herbal medicines at least once in their life time (Bhakuni *et al*, 1974; Farnsworth *et al*., 1985).

2.2.2.(b) Cytotoxicity assays

One of the most regular ways to examine cell viability and cytotoxic effects are through assessing cell membrane integrity due to the fact that potential cytotoxic compounds often compromise cell membrane integrity. Dyes such as trypan blue or propidium iodide are introduced to the inside of a healthy cell, however, if the cell membrane is compromised, the dye goes across the membrane and stains the intracellular components (Riss *et al*., 2004).

On the other hand, integrity of the membrane can be estimated by observing the pathway of substance that is usually hidden inside cells to the outside. Lactate

dehydrogenase (LDH) is commonly measured using the LDH leakage assay. It is used as an indicator for cytotoxicity. This assay is based on the evaluation of lactate dehydrogenase activity in extracellular medium and is regarded as simple, reliable and fast evaluation of cytotoxic activity (Decker & Lohmann-Matthes, 1988). Irreversible cell death due to cell membrane damage can be indicated by the loss of intracellular LDH into the culture medium. On the contrary, relative figures of live and dead cells within the same cell population can be identified using protease biomarkers. Healthy cell membrane is needed for active live-cell protease and therefore losses its activity if the membrane is compromised, where the protease uncovers to the external environment. Measurement of the dead-cell protease after the cells have lost membrane integrity can only be done in culture media as it is unable to cross the cell membrane (Niles *et al*., 2007).

MTT [3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2-tetrazolium bromide] assay can also be used to measure cytotoxicity. In this assay, the cell reducing potential can be measured using a colorimetric reaction. Viable cells are able to reduce the MTT reagent to a coloured formazan product. Correspondingly, a similar redox-based method was developed using fluorescent dye, resazurin. Likewise, researchers have also come up with an assay using ATP content as a viability marker (Riss *et al*., 2004). ATP is used as the limiting agent for the luciferase reaction in this bioluminescent assay (Fan & Wood, 2007).

The sulforhodamine B (SRB) assay is used to determine cell density based on the measurement of cellular protein content. Vichai & Kirtikara. (2006) have concluded that this assay is an efficient and highly-cost effective method for cytotoxicity screening as it required simple equipment, inexpensive reagents and is able to test a large number of samples within a few days. On the other hand,

metabolic activity-based WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) assay works similarly to MTT by reacting with the mitochondrial succinate-tetrazolium reductase forming the formazan dye. It produces water-soluble formazan rather than the water-insoluble product of the MTT assay and comparatively not as time consuming as the MTT assay (Ngamwongsatit *et al*., 2008).

As for the clonogenic assay, it is an *in vitro* cell surviving assay which is based on the ability of a single cell to grow into a colony. At least 50 cells are needed to define it as a colony. Importantly, this assay tests the ability of every cell to undergo unlimited division. It is the method of choice to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents (Franken *et al*., 2006). In addition, the kinetics of cytotoxic response can be examined through label-free real-time techniques based on electric cell-substrate impedance sensing (ECIS).

2.2.2.(c) **Importance of cytotoxicity assay**

Cytotoxicity testing has long been used to evaluate cell viability. However, comprehensive understanding on cytotoxicity estimation necessitates additional methods to evaluate short and long-term cytotoxicity. The degree of cytotoxicity is varied and depends on the methods used to evaluate it (Fellows & O'Donovan, 2007). If cytotoxicity testing were evaluated with different assays, the obtained data would be able to give more information and comprehensible idea of its mechanism. The need for new *in vitro* testing methods is great because the high failure rate (40-50%) of pharmaceutical drugs is due to toxicity (Sumantran, 2011) as well issues pertaining to animal ethics. Therefore, many researches channelled their interest in

finding cytotoxic agents through natural product research attributed by the ability to produce an array of chemical compounds.

2.3 Medicinal claims of herbal preparations

Interestingly, over the past years there are numerous claim by the proponents and consumers that both semalu and manna plus have been informally used in treatment of dengue patients, It is suggested to boost up platelet in patients with serious decline in platelets. (Asha Ravindran, 2013). Severe thrombocytopenia and increased vascular permeability are two major characteristics of dengue haemorrhagic fever. The commonly recommended dose regimens are two capsules of semalu and manna plus three times daily for a period of a week or two. In most cases, the subject continues this supplement for promotion of general health. There are claims that Semalu is used for genito-urinary tract hygiene, prevention of genito urinary tract infection (UTI), increase vaginal strength, used as an adjunct for the treatment of gall stones and renal stones, is a natural mild antibiotic, helps treat viral fever, improves genito urinary tract healing and is an adjunct treatment for acne (Mas Ayu Global, 2013). As for Manna plus it is believed to regulate the biochemical and physiological balance of the body, improving the quality and quantity of blood cells, optimisation of immunity and maintaining a disease refusal state, also regulates blood fats (lipoproteins), carbohydrate and protein metabolism and helps in maintaining kidney function (Mas Ayu Global, 2013).. This was mainly attributed to their ingredients which consist of these edible plants and spices which are known for their medicinal properties respectively. For instance, *A. Sativum* it possesses antimicrobial, antiprotozoal, antimutagenic, antiplatelet and antihyperlipidemic properties when consumed by humans. *A. cepa* is highly valued

for its therapeutic properties Research shows that onions may help guard against many chronic diseases. That's probably because onions contain generous amounts of the flavonoid quercetin (Aguirre *et al*., 2011). Studies have shown that quercetin protects against cataracts, cardiovascular disease, and cancer. *N. sativa*, commonly known as black seed or black cumin, are used in folk (herbal) medicine all over the world for the treatment and prevention of a number of diseases and conditions that include asthma, diarrhoea and dyslipidemia (Ali & Blunden, 2003). *C. longa* has several biological properties, including antioxidant activity, anti-bacterial and immunomodulating properties, water and fat soluble extracts of turmeric and its curcumin component exhibit strong antioxidant activity, comparable to vitamins C and E. *A. indica* consists of alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones; biologically most active compound is azadirachtin (Kumar & Navaratnam, 2013). *Z. officinale*, commonly known as ginger is an important kitchen spice and also possess a myriad health benefits. The rhizomes have been used since antiquity in the various traditional systems of medicine to treat arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, hypertension, dementia, fever, infectious diseases, catarrh, nervous diseases, gingivitis, toothache, asthma, stroke and diabetes (Zadeh & Kor, 2014). *S.*

Aromaticum the antimicrobial activity of clove essentials oil have been studied against a large number of multi-resistant *Staphylococcus epidermidis* and other strains of bacteria, It is well known previously, as an anti-carcinogenic, as a traditional remedy for asthma, disorder of digestive system, dental disorders, respiratory disorders, headaches and sore throat (Mahmoud *et al*., 2007). Besides the reported antimicrobial, antifungal and antiviral properties, the essential oil of S.

aromaticum shows anti-inflammatory and anaesthetic activities. The main constituent of clove essential oil is eugenol (Barakat, 2014).

However these claims warrant a more objective evaluation in terms of its pharmacological properties such as antioxidants, antibacterial, cytotoxicity and anti-thrombocytopenia. With this in view the following pre-clinical evaluation of semalu and manna plus pharmacological properties was undertaken.

2.3.1 Antibacterial activity

2.3.1.(a) Resistance towards bacteria

The discovery of antibiotics and their uses as chemotherapeutic agents paves the pathway to eradication of infectious disease caused by bacteria. Contrarily, antibacterial resistance has been a major threat causing public health concerns globally, further complicates the management of infections caused by bacteria, viruses, fungi and parasites (WHO, 2014). Exposure of pathogenic microbes to one or more antimicrobial agent causes genetic modification that transmits resistance. Emergence of multi-drug resistance bacterial strains on a global scale limits the effectiveness of drugs and hinders treatment of infections. Infections acquired by these resistance bacteria negatively prolong treatment period, cost, outcomes and disease spread (Okeke *et al*., 2005).

Antibacterial resistance is a natural phenomenon that's caused by genes that impart resistance known as environmental resistome. These genes could be transferred from non-disease causing bacteria to pathogenic bacteria, causing significant antibiotic cross resistance (Wright, 2010). Under continued selection pressure, selected resistant organisms multiply and spread to other geographic landscapes and microbes via transfer of resistance genes (Levy & Marshall, 2004).

Horizontal gene transfer via conjugation, transduction or transformation could be the possible ways of resistance gene transfer among these bacteria (Anderson, 2003). Resistance is the most commonly encountered problem with bacteria, where treating common infections are becoming alarmingly difficult in many parts of the world (WHO, 2014).

Mutation and adaptations caused bacteria to become resistant to certain drugs, which in turn causes newer infections. MRSA (methicillin or multi-resistant *S. aureus*), pneumococci resistant to penicillin and macrolides and VRE (Vancomycin-resistant *enterococci*) are example of multiple drug resistant bacteria (Nikaido, 2009). Serious concerns over the emergence of gram-negative pathogen resistant to most agents that were once essential are alarming (Livermore, 2004). The emergence of pan-resistant gram-negative strains, especially those of *P. aeruginosa* and *A. baumanii*, occurrence could attribute to lower or halting the production of newer antibacterial agents. Therefore limited or no agents are available to fight these strains. Researchers have established that drug resistant TB (tuberculosis) needs treatment regimens that use an array of antibiotics called fluoroquinolones and injectable medications, such as amikacin, kanamycin or capreomycin. Few types of TB are developing resistance to these medications as well.

2.3.1.(b) **Resistance in bacteria**

The search for solutions to this global crisis of new and re-emerging infectious disease caused by antibiotic resistance is on the rise. Many companies are focusing on research based on finding new antimicrobial compounds from a wide variety of resources available to mankind.

Global surveillance reports in 2014 on antibiotic resistance reveals that resistance is no longer prediction for the future but it's the present scenario around the world, hindering the ability to treat common diseases and infections. Without any warning or calculated plans the world is stepping into the post antibiotic era, where common and easily treatable infections and injuries could once again be life threatening (WHO, 2014). Few examples include cephalosporins failure to treat gonorrhoea, fluoroquinolones failure to treat urinary tract infection caused by *E. coli*, resistance to treat infections caused by *Staphlylococcus aureus* and carbapenem antibiotics inability to treat life-threatening infections caused by intestinal bacteria.

Reports from 10 countries suggest that gonorrhoea treatment have seen a major decline due to resistance to last resort treatments (third-generation cephalosporins) (WHO, 2014). Gonorrhoea could be life-threatening in coming decades as there are no available vaccines or antibiotic components in development. Infections caused by these drug resistant bacteria are fatal and lethal clinical manifestations could be a possibility. Patients infected with these bacteria consume more medication than patients that are infected with the same bacteria but aren't resistant.

2.3.1.(c) **Antimicrobial assay**

Broth microdilution is a commonly employed quantitative reference method in clinical laboratories and a useful technique for determining Minimum Inhibitory Concentration (MIC) of large number of test samples as described by, Eloff (1998) in this method, susceptibility panel in 96-well microtiter plates were containing various concentrations of antimicrobial agents. Then, fixed numbers of bacteria was inoculated into the wells of 96-well microtiter and incubate overnight at 35^0C. The

MIC value was observed as the lowest concentration where no viability was observed in the wells of 96-microwell plates after incubation.

A dye such as p-iodonitrotetrazolium (p-INT) is used as an indicator of microbial growth through colorimetric variations (color changes) and turbidity changes. Underlying principle behind it is, during active bacterial growth, an electron is produced and transferred to the dye forming a colored formazan. In the presence of an antimicrobial agent these formazans are inhibited (Masoko & Eloff, 2005). Dyes eliminate the need for spectrophotometric analysis. The lowest concentration of semalu and manna plus extracts was defined as its MIC values, which facilitated complete inhibition of microbial growth (Souza *et al*., 2005).

It is a generally used strategy, taking into consideration the simultaneous testing of different antimicrobials effortlessly especially when commercially prepared microtiter plates are utilized (Eloff *et al*., 1998). In comparison to disc diffusion, agar dilution and broth microdilution are found to beat a few restrictions of the disc diffusion technique, essentially that capacity to reach quantitative inference by deciding the MIC value for antimicrobials (Kim & Kim, 2007). Accordingly, both gar dilution and broth microdilution are helpfully utilized for routine antimicrobial susceptibility testing in clinical research facility. Nevertheless, impediments of the technique essentially are connected with the absence of or poor development of numerous anaerobic microorganisms. Testing a few fastidious anaerobes gives inconsistent and unreliable results in light of poor development of strains because of excessive exposure to oxygen amid the set-up procedure (Clinical and Laboratory Standard Institute, 2009).

2.3.2 Antioxidant activity

Free radicals, in particular responsive oxygen species (ROS) and receptive nitrogen species (RNS), are known to harm lipids, proteins, compounds and nucleic acids prompting cell or tissue damage involved during the time spent maturing. Extensive variety of degenerative illnesses including irritation, disease, atherosclerosis, diabetes, liver damage, Alzheimer, Parkinson, and coronary heart pathologies are because of these free radicals and oxidative anxiety. A few proofs demonstrate that oxidative anxiety can prompt cell and tissue damage. The term oxidative anxiety demonstrates that the cancer prevention agent status of cells and tissues is adjusted by presentation to oxidants. There happens consumption of cell reinforcements amid oxidative anxiety. The ROS and RNS incorporate various responsive substances specifically superoxide O_2 –. hydroxyl (OH.), peroxyl (ROO.), peroxynitrite (.ONOO.) and nitric oxide (NO.) radicals and also without non radical species as hydrogen peroxide (H_2O_2), nitrous corrosive (HNO_2) and hydrochlorous corrosive (HOCl) (Mavi *et al*., 2004; Mosquera *et al*, 2007).

Interestingly, oxygen consuming living being produced cell reinforcement barrier systems that capture the harm brought about by ROS and RNS elements. The resistance component can be both enzymatic and non-enzymatic. In the enzymatic instruments, a few compounds, for example, superoxide dismutase, catalase, glutathione reductase, peroxidase and nitric oxide synthase are included. While non enzymatic components are confined to cell reinforcements and catching specialists, for example, ascorbic corrosive, α–tocopherol, β–carotene, glutathione, flavonoids, uric corrosive, cysteine, vitamin K, serum egg whites, bilirubin, and follow components, for example, zinc and selenium (Mosquera *et al*., 2007).

2.3.2.(a) **Free radicals**

A radical (all the more decisively, a free radical) is a particle, atom, or particle that has unpaired valence electrons or an open electron shell, and in this manner might be seen as having one or additionally "dangling" covalent bonds. With a few special cases, these "dangling" bonds make free radicals exceptionally synthetically receptive towards different substances, or even towards themselves. Their atoms will regularly dimerize or polymerize if they interact with one another. Most radicals are stable just at low concentration in inert media or in a vacuum. A prominent case of a free radical is the hydroxyl radical (HO•), a particle that is one hydrogen atom shy of a water atom and in this manner has one bond "dangling" from the oxygen. Two different cases are the carbene particle (CH 2), which has two dangling bonds; and the superoxide anion (•O−2), the oxygen atom O_2 with one additional electron, which makes them dangle bond. Interestingly, the hydroxyl anion (HO−), the oxide anion (O2−) and the carbenium cation (CH^{+3}) are not radicals, since the bonds that might have all the earmarks of being dangling are indeed determined by the addition or deletion of electrons. Free radicals assume an imperative part in various natural procedures. Large portions of these are necessary for life, for example, the intracellular killing of microscopic organisms by phagocytic cells, for example, granulocytes and macrophages. Scientists have likewise embroiled free radicals in certain cell signalling processes, known as redox signalling. The two most essential oxygen-focused free radicals are superoxide and hydroxyl radical. They get from sub-atomic oxygen under diminishing conditions. Notwithstanding, due to their reactivity, these same free radicals can take an interest in undesirable side responses bringing about cell harm. Free radicals assume an essential part in various organic procedures. Over the top measures of these free

radicals can prompt cell damage and passing, which might add to numerous infections, for example, growth, stroke, myocardial dead tissue, diabetes and significant issue (Karthikeyan *et al*., 2011). Numerous types of tumour are thought to be the consequence of responses between free radicals and DNA, conceivably bringing about changes that can antagonistically influence the phone cycle and possibly prompt danger (Mukherjee *et al*., 2004). A portion of the indications of maturing, for example, atherosclerosis are additionally ascribed to free-radical actuated oxidation of cholesterol to 7-ketocholesterol (Lyons & Brown, 1999). Moreover free radicals add to liquor affected liver harm, maybe more than liquor it. Free radicals delivered by tobacco smoke are embroiled in inactivation of alpha 1-antitrypsin in the lung. This procedure advances the improvement of emphysema.

2.3.2.(b) **Antioxidants and its mechanism**

Antioxidants are multifaceted; their uses are not only restricted to biological species but have a wide scope of application. Antioxidants, based on biologically relevant terms are defined as a substance that significantly inhibits the damaging effects of oxidation in animal tissue. Scavenging of reactive oxygen species and prevention of radical chain reaction or radical production are characteristics of antioxidants (Huang *et al*., 2005)

Hydrogen Atom Transfer **(**HAT) and Single Electron Transfer (SET) are two important antioxidant mechanisms involved in deactivating radicals. The outcome regardless of mechanism is similar, but kinetics and potential side chain reaction differ. Proton-coupled electron transfer and HAT reactions might take place simultaneously, but the mechanism that dominates in a particular system is based on the structure and properties of antioxidants and solvent system used. Efficacy of

antioxidants and its mechanism depends on factors such as bond dissociation energy (BDE) and ionization potential (IP) (Wright *et al*., 2001).

HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation (AH) any H donor)

$ROO^{\bullet} + ArOH = ROOH + ArO^{\bullet}$

The above reaction shows hydrogen atom transfer reaction. Through aromatic ring stabilization the aromatic radical remains stable giving out a hydro peroxide as the by product. Inhibition of the peroxy radical takes place with the addition of antioxidant to the system through hydrogen transfer, followed by SET pathway of antioxidant pathway. BDE of the H-donating group in the potential antioxidant determines the relative reactivity of in HAT. Reactions are rapid, are solvent and pH independent. Reducing agents such as metal could cause major complications in HAT assays (Wright *et al*, 2001).

SET-based methods are primarily based on the ability of the antioxidant to donate an electron to stabilize the free radicals.

$ROO^{\bullet} + ArOH\ ROO^{-} + ArOH+^{\bullet}$

$ROO^{-} + ArOH+^{\bullet}\ ArO^{\bullet} + ROOH$

The peroxy radical is stabilized based on the electron donated by the antioxidant molecule. Due to an abundance of electrons on the oxygen a negative peroxy radical formation occurs. Through the HAT process the positively charged aromatic free radical donates the (H+) proton. The two mechanisms most occur in tandem in all samples, with the balance determined by structure and pH. SET reaction are pH specific, with increase in pH its IP decreases, reflecting in increased

electron donating potential (Huang *et al*., 2005 & Wright *et al*., 2001). SET reactions are time consuming and slow, its antioxidant capacity is calculated based on percent decrease in product. Variability, reproducibility and consistency of results can be affected by contaminants and trace components (metals) interrupt SET (Prior *et al*., 2005).

There are several synthetic antioxidants available in the market today, such as butylated hydroxytoulene and butylated hydroxyanisole, these products could manifest an array of side effects, and so its safety is not established completely. Hence the search for natural antioxidants is utmost priority (Alma *et al*., 2003).

2.3.2.(c) **Antioxidant assay**

In this dissertation the antioxidant properties of ethanol and methanol extracts of Semalu and Manna Plus were evaluated through DPPH free radical scavenging, total flavonoid and total phenolic content assays.

2.3.2.(d) **2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay**

Most commonly used method for screening natural products for the presence of antiradical compounds is test based on free radical scavenging of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. This stable free radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule in solution DPPH (Kroyer, 2004). In the presence of a free radical scavenger the intense purple color that change into pale yellow indicating free radical scavenging is measured at 517 nm (Blois, 1958). Disadvantages of spectrophotometric tests are that they measure the extract as a whole without any indication of the most potent free radical scavengers. *In vitro* techniques based on DPPH$^{\bullet}$ scavenging have been recently