Investigation of *In-vitro* Antioxidant and Cytotoxic Activity of *Blumea lacera* Leaf Extract

By

Mujtaba Ali Khan
15346039

A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

Mujtaba Ali Khan
15346039

Approval

The thesis titled "Investigation of *In-vitro* Antioxidant and Cytotoxic Activity of *Blumea lacera* Leaf Extract" submitted by Mujtaba Ali Khan (15346039) of Summer, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on 10th October, 2019.

Examining Committee:

Supervisor:
(Member)

Dr. Raushanara Akter
Associate Professor, Department of Pharmacy
Brac University

Program Coordinator:
(Member)

Dr. Hasina Yasmin
Professor, Department of Pharmacy
Brac University

Departmental Head:
(Chair)

Dr. Eva Rahman Kabir
Chairperson, Department of Pharmcy
Brac University

Ethics Statement

This study does not involve any kind of animal or human trial.

Abstract

Blumea lacera (family: Asteraceae) is a very potent plant for its various therapeutic properties. This research was intended to detect the antioxidant and cytotoxic potential of chloroform extract of this plant leaf. Antioxidant potential was assessed by a number of *in-vitro* antioxidant studies and among them DPPH free radical scavenging assay showed 86.43% inhibition of free radicals at 1200µg/mL of the extract with IC_{50} of 2.39µg/mL. Highest total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) was found to be 285.47mg of GAE/g, 287.12mg of QE/g and 272.21mg of AAE/g at 1200µg/mL of extract, respectively. Cytotoxic activity was measured using *in-vitro* MTT assay against HeLa and MOLT-4 cell lines that showed IC_{50} of 3.46mg/mL and 3.29mg/mL for the extract. These results showed that the extract had strong antioxidant and low cytotoxic potential.

Keywords: antioxidant; cytotoxic; *in-vitro*; free radical; phenolic; flavonoid

Dedication

Dedicated to my family members for their constant support.

Acknowledgement

First of all, all the glory and praise of Almighty Allah for all the generosities awarded to me to complete this project. Secondly, I would like to thank my family for their constant mental support and my project supervisor, Dr. Raushanara Akter, Associate Professor, Department of Pharmacy, Brac University.

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List of acronyms

WHO	World Health Organization
API	Active Pharmaceutical Ingredient
ROS	Reactive Oxygen Species
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
SOD	Superoxide Dismutase
8-OH-Gua	8-hydroxyguanine
AD	Alzheimer's Disease
PUFAs	Polyunsaturated Fatty Acids
MCI	Mild Cognitive Impairment
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
TBHQ	Tertiary Butyl Hydroquinone
GSH	Glutathione
GSSG	Oxidized Glutathione
GPx	Glutathione Peroxidase

CAT	Catalase
DPPH	1,1-diphenyl-2-picrylhydrazyl
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
TAC	Total Antioxidant Capacity
ECM	Extracellular Matrix
CAFs	Cancer-Associated Fibroblasts
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
FMPs	Folk Medicinal Practitioners
TMPs	Tribal Medicinal Practitioners
BL	*Blumea lacera*
AA	Ascorbic Acid
AAE	Ascorbic Acid Equivalent
GAE	Gallic Acid Equivalent
QE	Quercetin Equivalent
MAE	Microwave Assisted Extraction
SFE	Supercritical Fluid Extraction
LAF	Laminar Air Flow

Chapter 1

Introduction

1.1 Importance of medicinal plant

1.1.1 Medicinal plant as a source of lead compound

Plants have been important for several thousands of years in clinical purposes in which most of people still using herbal medicine to meet their health needs (Bókkon, 2012; D. M. Brown et al., 2004). The fundamental construction and purity of the original plant is reserved by the conventional use of phytotherapies in order to use the whole plants or a portion of its minimally spurious components as medicinal intentions (Falzon & Balabanova, 2017). Since days of yore, humankind has scanned for curing compounds to expel ache and fix different sicknesses (Rafieian-Kopaei & Sewell, 2014).

Medicinal plants can be described as “a group of plants that possess some special properties or virtues that qualify them as items of drugs and therapeutic agents, and are useful as articles of medical management and treatment of diseases” (Ghani, 2003d). According to WHO, among the whole population, about 80% of individuals get benefited from medicinal plants in the ailment of disease (Süntar, 2019). In case of traditional medicine, the whole plant was not used as medication, on the other hand, in case of allopathic medicine along with the API, suitable excipients were added to make a dosage form through a number of trial and error on animals and humans (F. S. Li & Weng, 2017).

Now-a-days, to establish a medicine from specific parts of medicinal plants, it involves rigorous inquiry into a plant's biological origin, classification, and macroscopic and microscopic features (Hu et al., 2019). Most of the medicinal plants contain biologically

active compounds such as essential oils, flavonoids, glycosides, polyphenols, alkaloids, tannins, saponins that play a vital role in disease treatment (Iwanowicz-palus et al., 2019).

Lead optimization and structural modification are used to synthesize new drug (Shamas-Din & Schimmer, 2015). A lead compound is a chemical compound which is expected to be therapeutically helpful in pharmacological or biological activities but which still has a sub optimum composition which needs changes to match properly into the objective (Cragg & Newman, 2013). The synthesis of an biologically active compound is to be optimized to meet every clinically useful requirement of stereo electronic, physicochemical, pharmacokinetic and toxicological compounds (Patidar et al., 2011). Through chemical modification and lead optimization, various drugs are discovered. Some examples are given in **Table 1**.

Table 1: Isolation of some natural drugs.

Lead compound/ Drug	**Chemical class**	**Source**	**Medical use**	**Mechanism of action**	**Reference**
Taxol	Alkaloid	*Taxus brevifolius*	Anti-neoplastic	Interference with the normal function of microtubules during cell division.	(Ahmad Khan & Ahmad, 2019)
Saffron	Phenols	*Crocus sativus* L	Anti-depressant	Inhibiting synaptic serotonin reuptake.	(Srivastava et al., 2019)
Irinotecan	Alkaloid	*Camptotheca acuminata*	Anti-cancer	Topoisomerase I inhibitor.	(Tewari et al., 2019)
Aescin	Saponin	*Aesculus hippocastanum*	Anti-inflammatory	Serotonin antagonism and histamine antagonism.	(Oladunni Balogun et al., 2019)
Topotecan	Phenols	*Camptotheca acuminata*	Anti-cancer	Prevents topoisomerase-I from re-ligating the nicked DNA strand.	(Inoue et al., 2019)

Due to the increasing acceptance of natural products, the need for medicinal plants is increasing in both developed and developing nations (Das et al., 2019). The wide chemical range of secondary metabolites and their intrinsic capabilities find possibilities for fresh drug discovery (Marchi et al., 2012). Isolated plant metabolites such as alkaloids, phenols and terpenes were used through numerous changes in the primary fundamental skeleton of products and drugs (Gupta et al., 2019). Some examples of bioactive compounds and their common pharmacological activities are listed in **Table 2**.

Table 1: Some bioactive compounds and their common pharmacological activities (Segneanu et al., 2017).

Compound type	Pharmacological properties
Terpenoid	Antimicrobial, antiviral, antiviral, anthelmintic, antibacterial.
Phenolic acids	Anticarcinogenic and antimutagenic, anti-inflammation.
Alkaloids	Antispasmodic, antimalarial, analgesic, diuretic activities.
Flavonoids	Antioxidant, cardiovascular protective, anti-inflammatory.
Saponins	Antitumor, antiviral, antifungal, anti-inflammatory, immunostimulant.
Tannins	Antioxidant, anti-carcinogenic, diuretics, hemostatic, anti-mutagenic.

1.1.2 Traditional use of medicine as a means of remedy

For disease therapy, it is evident that there are about 70,000 plant species used and for their medical use only about 15 percent of the plant species cultivated in the globe is investigated (Süntar, 2019). Despite this small level, 25% of standard medicinal products currently used in contemporary medicine are of plant origin. Currently, approximately 120 distinct chemical substances are obtained from the various crops with therapeutic properties and are therefore used worldwide as drugs (Sen & Samanta, 2014). Primary metabolites like starch, glucose, protein, polysaccharide, nucleic acids and lipids are useful to human body growth and development (Ahmad Khan & Ahmad, 2019). Secondary metabolites such as- alkaloids,

flavonoids, saponins, terpenoids, steroids, glycosides, tannins, volatile oils show medicinal and therapeutic properties when present in certain amounts in plants. For example, *Curcuma longa* contains the phyto-constituent- curcumin C_3 complex, which is used against inflammatory diseases like arthritis (Alelign et al., 2014).

Some examples of medicinal plants in Bangladesh with their pharmacological activity and traditional use are given in **Table 3**.

Table 2: Some examples of traditionally used medicinal plants in Bangladesh (Eatimony et al., 2019).

Scientific Name (Family Name)	Local Name	Used Plant Parts	Pharmacological Activity	Traditional Use
Catharanthus roseus G. Don (Apocynaceae)	Nayan tara	Leaf, flower	Cytotoxic	Diabetes, cancer.
Eclipta prostrata L. (Asteraceae)	Kalahuta, Kalokeshi	Leaf, root, whole plant	Antiseptic	Bleeding from external cuts and wounds, skin infections, toothache.
Ricinus communis L. (Euphorbiaceae)	Venna	Leaf, seed oil	Anti-inflammatory	Inflammation.
Cinnamomum camphora (L.) J. Presl (Lauraceae)	Korpur	Leaf	Anti-epileptic	Constipation, paralysis, epilepsy, spasms.
Cinnamomum verum J. Presl (Lauraceae)	Daruchini	Inner and outer bark, leaf	Antioxidant	Digestive disorder, diarrhoea, flatulence, nausea.

Antibacterial, antineoplastic, antifungal, anti-atherosclerotic, antioxidant and antidiabetic characteristics of plant raw materials are because of the presence of biologically active substances like- alkaloids, terpenoids, flavonoids, tannins and sterols (Iwanowicz-palus et al., 2019).

Catharanthus roseus

Eclipta prostrata

Ricinus communis

Cinnamomum camphora

Figure 1: Some traditionally used medicinal plants.

1.2 Free radicals and reactive oxygen species (ROS)

A free radical is an atom or molecule with at least one unpaired electron (Pan et al., 2019; Ramos-Tovar & Muriel, 2019). In human body, unpaired electron of free radicals enables them to search for and collect electrons from other molecules (Mittler et al., 2011; Shah & Shafi, 2019). In nearly all eukaryotic cells, ROS are generated. They respond with lipids, proteins and nucleic acids when available in excess resulting in comprehensive tissue impairment and injury (Griendling et al., 2016). ROS is produced through consecutive single-electron reductions, generating superoxide ($O_2^{\cdot-}$),hydroxyl ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) radical (Van Acker & Coenye, 2017).

1.2.1 Occurrence of ROS and free radical reaction

During different cellular metabolism and for a multitude of functions such as initiating apoptosis, repair process, metabolizing, xenobiotics, antioxidant stimulation, signalling, free radicals are generated from oxygen, that leads to its unavoidable manufacturing in animal cell (Shrivastava et al., 2019). Superoxides, hydrogen peroxides and radical hydroxyl trigger multiple kinds of intracellular harm, which are frequently referred as free radicals (Hong et al., 2019).

Free radicals can be categorized as shown in **Figure 2** based on the kinds of free radicals generated as a consequence of metabolic reaction (Sharifi-Rad et al., 2018).

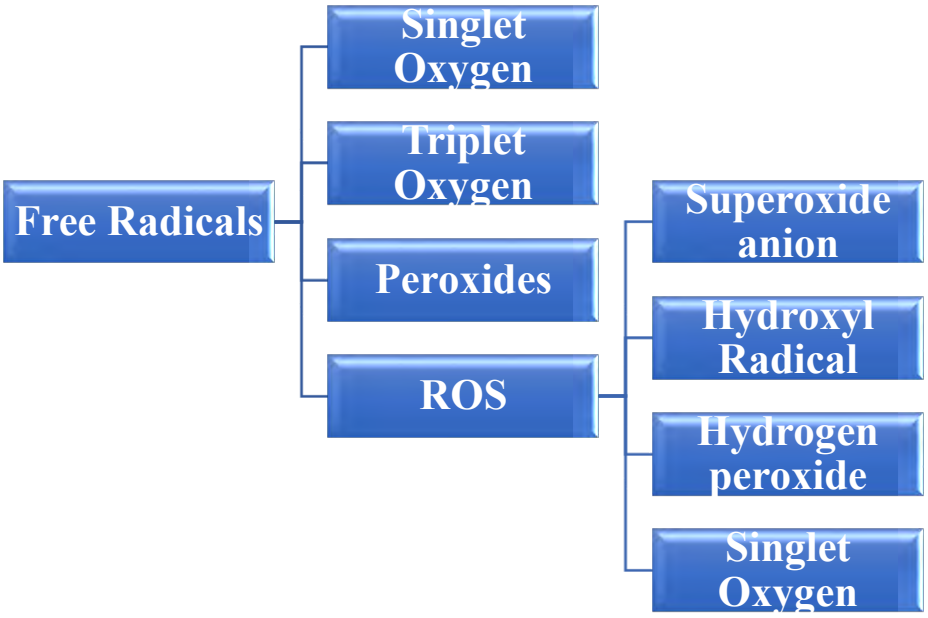


Figure 2: Classification of free radicals by formation.

Based on their sources, these free radicals can be further split into-exogenous sources, which is radiation, cigarettes smoke, heavy or transition, and, endogenous sources, which are produced through activation of immune cells, aging, ischemia, excessive exercise, mental stress, infection, cancer, inflammation (Lobo et al., 2010; Pham-Huy et al., 2008). Free

radicals are engaged in numerous physiological functions such as gene expression regulation, angiogenesis *etc.* (Yaribeygi et al., 2018).

1.2.2 Diseases caused by free radicals

Oxygen-free radicals are thought to be engaged in the occurrence of many diseases, as they are extremely reactive. Formation of increased free radical can cause harm to DNA other proteins, leading to disease complications (Stanjek-Cichoracka et al., 2018).

Table 3: Common ROS and free radicals-related diseases (Kehrer & Klotz, 2015).

Exogenous Sources	**Endogenous Sources**
Asbestosis	Alzheimer's disease
Emphysema	Amyotrophic lateral sclerosis
Atherosclerosis	Down's syndrome
Photic retinopathy	Parkinson's disease
Contact dermatitis	Autoimmune nephrosis: inflammation
Porphyria	Hemochromatosis
Protoporphyrin photooxidation	Werdnig–Hoffman disease

1.3 Mechanism of free radicals to cause disease

1.4.1 Oxidative stress

Oxidative stress is described as a situation in which the cell's antioxidant scavenging mechanism is disrupted by overproduction of ROS, leading in a paradoxical state of oxygen, requiring free radicals for cellular procedures but also interfering with vital metabolic processes at enhanced levels (Bisht et al., 2017; Sies, 2015; Valko et al., 2007). Oxidative stress causes cell integrity and tissue functions to deteriorate and encapsulates the pathogenesis of various illnesses (Balaban et al., 2005; Harman, 1956). Oxidative stress can

in turn lead to carbonyl stress where extremely reactive organic molecules capable of modifying proteins non-enzymatically, are created by oxidative damage to the surrounding tissues (Kirkham & Barnes, 2013).

1.4.1.1 Body and tissue damage through free radical and oxidative stress

Oxidative stress is the main pathogenic method that involves cell apoptosis or necrosis that ultimately causes tissue injury or cell death in the pathogenesis of the different biological systems (Adly, 2010; Kruk et al., 2019). Damage to DNA and RNA, lipid peroxidation, damage to protein, and degradation of carbohydrates are the hazardous responses of free radical (Hall & Braughler, 1989; Milne et al., 2005; Scandalios, 2005; Smith et al., 2013; Ziech et al., 2010).

Free radicals can have harmful effects on lipids through oxidative stress (Butterfield et al., 2006; Keller et al., 2005; Lovell et al., 1995). In case of Alzheimer's Disease (AD), the brain is abundant in phospholipids that are important to neurotransmission mechanisms as well as the basis for neuronal relations and cognition (Montine et al., 2012; Praticò et al., 2000; Roberts et al., 1998; Singh et al., 2010). Brain phospholipids, particularly docosahexaenoic acid and arachidonic acid, comprise a large percentage of polyunsaturated fatty acids (PUFAs) (Chen & Zhong, 2014). It has been discovered that the content of PUFAs in the brain is gradually declining as free radical manufacturing rises (Skoumalová & Hort, 2012; Söderberg et al., 1991).

Amino acids undergo oxidative damage, both free and within proteins (Sena & Chandel, 2012). Direct oxidation of the end branches results in the creation of carbonyl groups (aldehydes and ketones) and is particularly susceptible to assault by proline, arginine, lysine and threonine (Burton & Jauniaux, 2011; Dalle-Donne et al., 2003).

DNA is primarily targeted by OH· radicals, and interactions with either the DNA bases or deoxyribose sugars can generate a range of products (Halliwell & Gutteridge, 1999). For instance, OH· may generate 8-hydroxy-2'-deoxyguanosine by adding to guanine, which can be biochemically evaluated and immunohistochemically identified.

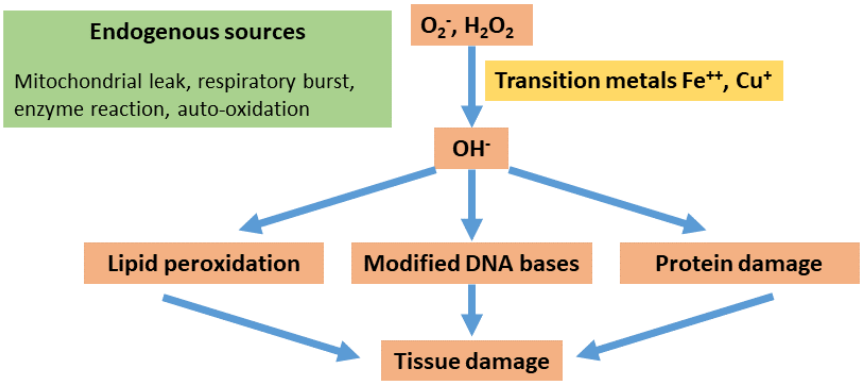


Figure 3: Schematic diagram of oxidative tissue damage by free radicals.

Figure 3 shows the schematic pathway of tissue damage caused by free radicals that is the result of damage to lipid, DNA and protein.

1.5 Antioxidants

Antioxidants are merely chemicals that can engage in redox reactions, typically serving as suppliers of electrons or reductants and/or fragmenting unstable molecules into comparatively inert products (Aitken et al., 2019). Antioxidant capacity can be achieved by giving an electron to the reactive oxidant (e.g., ROS), thus eliminating additional oxidative processes (Gutteridge & Halliwell, 2018; Walczak-Jedrzejowska et al., 2013). As a result, first cells should establish antioxidant capabilities to safeguard themselves from oxidative damage (Apak, 2019).

1.5.1 Type of antioxidants

Antioxidants are divided into primary or natural antioxidants and secondary or synthetic antioxidants two based on their function (Hamid et al., 2010). Primary or natural antioxidants

are the bond separating antioxidants that interact and transform them into more stable products with lipid radicals (Haida & Hakiman, 2019; Hamid et al., 2010). This class of antioxidants is primarily phenolic in structures and includes minerals, vitamins and phytochemicals (Hurrell, 2003). On the other hand, secondary or synthetic antioxidants stop the chain reactions and are mainly phenolic (Hurrell, 2003; Mehta & Gowder, 2015). For example, butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butyl hydroquinone (TBHQ) *etc.* They can also be classified alphabetically, like- antioxidant C contains carotenoids, antioxidant G contains glutathione *etc.* (Flora, 2009).

There are six classifications of antioxidants when divided kinetically (Flora, 2009). These are given in **Table 5**.

Table 4: Classification of antioxidants kinetically.

Mechanism of action	**Antioxidant examples**
Chain rupturing by antioxidants by reacting with radicals of peroxide with fragile N-H and O-H bonds.	Phenol, hydroquinone, aromatic amines
Antioxidants breaking bonds by interacting with alkyl radicals.	Quinones, nitrones, iminoquinones
Antioxidants decomposing hydrogen peroxide	Sulphide, phosphide, thiophosphate
Antioxidants deactivating metal.	Diamines, hydroxyl acids
Ending the cyclic chain by antioxidants.	Aromatic amines, nitroxyl radical
Several antioxidants act synergistically.	The group of phenolics in phenol sulfide reacts to hydroperoxide and peroxyl sulphide radical.

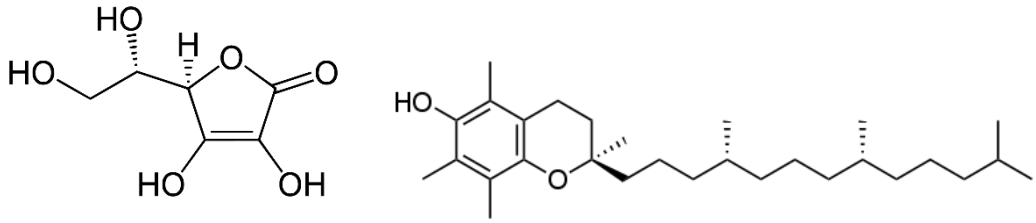
The category of antioxidants, either enzymatic or nonenzymatic antioxidants, relies on their form of catalytic action (Haida & Hakiman, 2019). The enzymatic antioxidants have some cofactors and appear to be extremely sensitive to reactive substrate species (Mehta & Gowder, 2015). On the other hand, non-enzymatic antioxidants vary from the preceding

because they do not have any particular substrate and could therefore nullify the RNS and ROS adverse impacts (Haida & Hakiman, 2019).

1.5.2 Different role of phytochemicals as antioxidants

Nutritional sources such as fruits, spices, vegetables, herbs, tea, etc. contain various kinds of antioxidants (Suleman et al., 2018). They function as free radicals scavengers in animal bodies (Mandal et al., 2009; Taira et al., 2015). Citrus fruits are source of vitamin C and contain a big quantity of natural antioxidants with many physiological advantages (Suleman et al., 2018). Tocopherols, acrobats and carotenoids are very well-known examples of antioxidants that play a key role in chronic infection therapy (Gomes et al., 2013). Carotenoids such as lycopene and lutein, vitamins (C, E), β-carotene and carotene are among the other antioxidants (Boskou et al., 2005). Flavonoids in crops safeguard them from various environmental pressures, while they act as an anti-cancer, anti-viral, anti-aging and anti-inflammatory agent in humans (Havsteen, 1983; Y. Li et al., 2017; Wang et al., 2018).

Various tissues have produce antioxidant systems for manipulating free radicals, catalysts for lipid oxidation, intermediate oxidation, and yields for secondary collapse (Agati et al., 2007; J. E. Brown & Kelly, 2007; Iacopini et al., 2008). These antioxidant compounds consist of phenolic acids, carotenoids, tocopherols, flavonoids capable of obstructing oxidation caused by Fe^{+3}, cleaning free radicals and serving as metabolites (Khanduja & Bhardwaj, 2003; Ozsoy et al., 2009).



Ascorbic acid (Vitamin C) α- Tocopherol (Vitamin E)

Figure 4: Some antioxidant phytochemicals.

1.5.3 Production of antioxidants in our body and their defense system

Cell, tissue and extracellular matrix exposure to the detrimental impacts of free radicals creates a sequence of responses and induces the opening of various inner defense mechanisms that eliminate free radicals and their components (Birben et al., 2012; Fernndez-Mej et al., 2013). The processes are (Mirończuk-Chodakowska et al., 2018):

a) **Preventive:** As a first line of defense, preventing free radicals and their derivatives from exposure to biological substances in the body.
b) **Repairing:** Involvement of a radical oxidation reaction disruption.
c) **Inactivation:** Free radical reaction products and their derivatives are inactivated by repairing or eliminating structural damage (Wu et al., 2013).

Endogenous antioxidants can be enzymatic or non-enzymatic, which are products of the metabolism of the body (Hrycay & Bandiera, 2015). One of the enzymatic antioxidants in the first line of defense that plays a significant part is SOD (Ardolino et al., 2011). Hydrogen peroxide and molecular oxygen is formed from superoxide anion by a disproportionate reaction, catalyzed by SOD (Mirończuk-Chodakowska et al., 2018). Glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and peroxiredoxins (Prxs) are other essential enzymatic antioxidants in the first line of defense (Hosokawa et al., 2007). These enzymes eliminate the hydrogen peroxide, producing water (catalase glutathione peroxidase) and molecules of oxygen (catalase) (Mirończuk-Chodakowska et al., 2018). The non-enzymatic compounds involved in the first line of defense belong to preventive antioxidants, and transferrin, ferritin, albumin and ceruloplasm are represented in blood plasma (Birben et al., 2012). By binding transition metal ions (e.g. iron and copper), these proteins prevent the development of fresh reactive species (Babula et al., 2012).

1.6 *In-vitro* study methods of antioxidant activity

On the basis of a single antioxidant test model, antioxidant activity should not be concluded. As a result, in practice, several *in-vitro* test methods are performed with the samples of concern to evaluate antioxidant activities (Alam et al., 2013).Several in-vitro antioxidant activity assays are available. For example- total flavonoid content (TFC), total phenolic content (TPC), DPPH free radical scavenging assay, total antioxidant capacity (TAC), ABTS radical scavenging assay, ferric reducing antioxidant power *etc*. Among these DPPH free radical scavenging assay is most reliable and a sophisticated technique and can be considered as the single assay for confirmation of antioxidant activity (Badarinath et al., 2010).

1.6.1 DPPH free radical scavenging assay

In natural antioxidant research, the DPPH assay has recently become quite common. One reason for this is that it is easy and extremely delicate technique (Moon & Shibamoto, 2009). In the **Figure 5**, the molecule 1,1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl; DPPH) is characterized by the delocalization of the extra electron over the molecule as a whole and as a stable free radical, so that the molecule does not dimerize like most other free radicals (Alam et al., 2013). Electron delocalization also provides rise to the deep violet color, characterized by an ethanol or methanol solution absorption band centered at about 517 nm. Violet color is lost when a DPPH solution is mixed with the sample of interest that can donate a hydrogen atom (Alam et al., 2013). The antioxidant activity in samples is proportional to the DPPH radical's loss (Moon & Shibamoto, 2009).

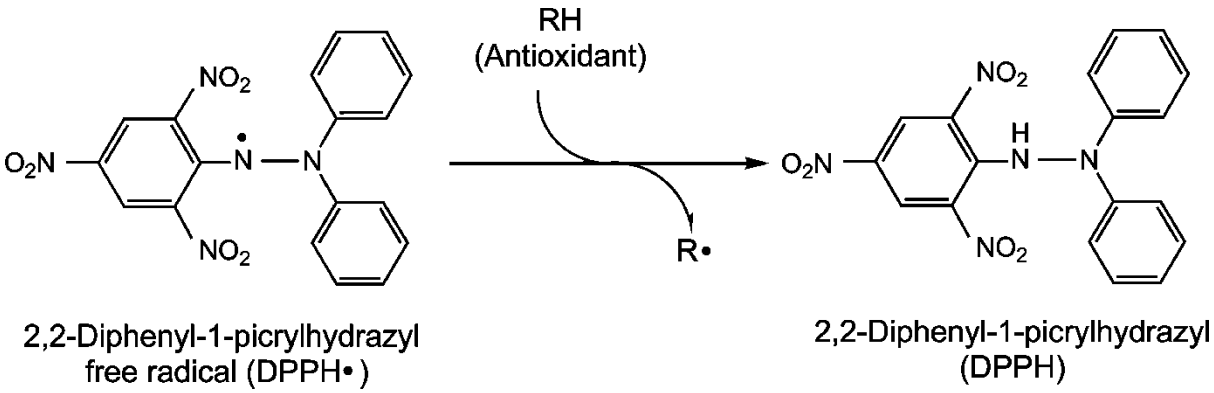


Figure 5: Reaction between DPPH radical and antioxidant to form DPPH (Moon & Shibamoto, 2009).

1.7 Cancer

Cancer is a significant problem for public health globally and is one of the leading causes of death ranked second in developed nations after cardiovascular disorders (Aqil et al., 2019; Tlsty & Coussens, 2006). Cancer is said to be the cluster of illnesses characterized by uncontrolled development and metastasis of uncommon or abnormal cells (Tewari et al., 2019). Cancer in humans is a multi-stage method involving different genetic or epigenetic modifications that eventually drive the ordinary cell's malignant transformation **(Figure 6)** (Aqil et al., 2019).

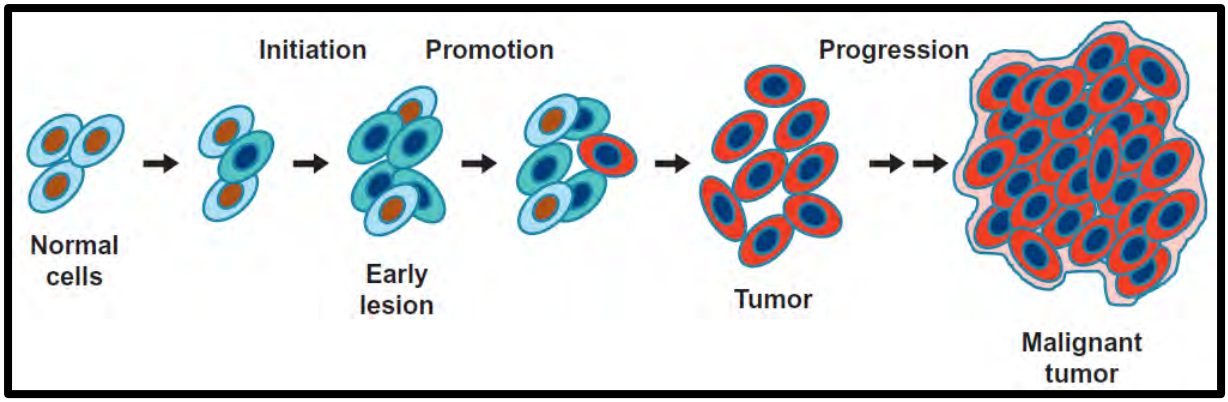


Figure 6: The development of cancer cells (Aqil et al., 2019).

1.7.1 Pathogenesis of cancer

The present predominant opinion of cancer is that of a condition involving irreversible genomic change— changes involving single gene mutations, or modification, amplification, or loss of big genome segments (Albertson et al., 2003; Tlsty & Coussens, 2006). In critical gatekeeper genes, such as oncogenes and tumor suppressor genes dominant gain-of-function and recessive loss-of-function changes (Levental et al., 2009; Marsh et al., 2013). Thus, in conjunction with those influencing extrinsic programs such as immune response, matrix metabolism, tissue oxygenation, and vascular status, genomic changes influencing inherent cellular programs such as control of the metabolism, programmed cell death, cell adhesion, cell cycle, and differentiation causes the progression of human cancer (Tlsty & Coussens, 2006).

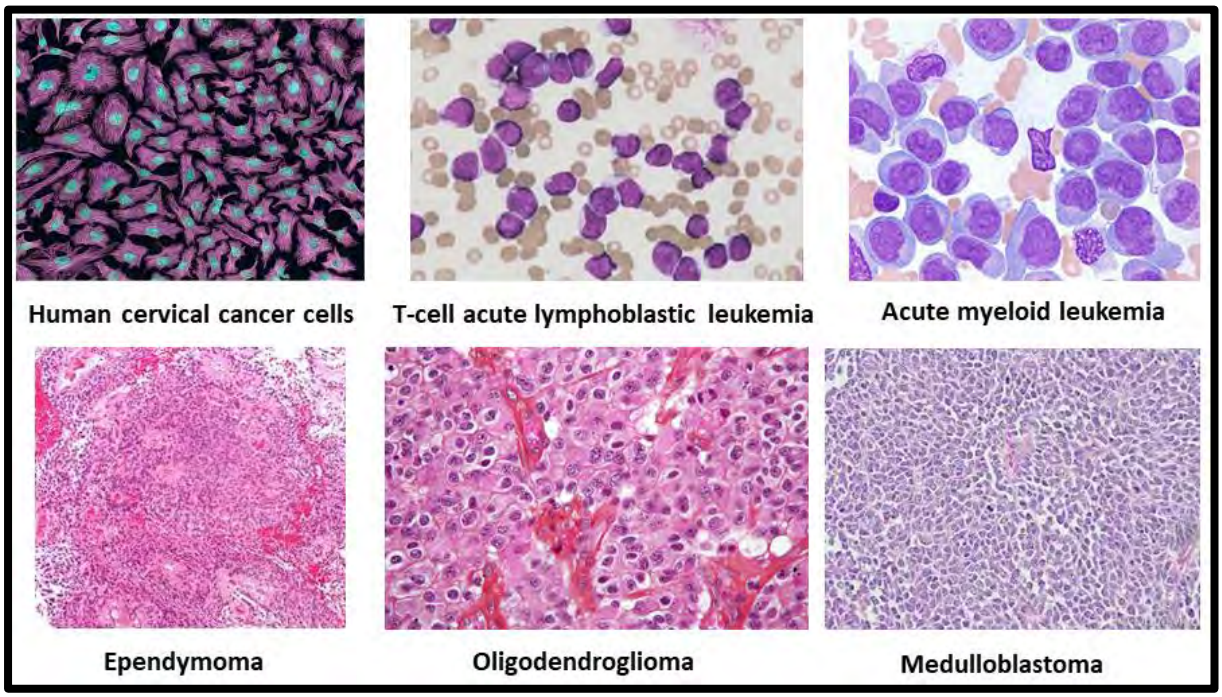


Figure 7: Different types of cancer cells.

1.7.2 Worldwide cancer prevalence

Cancer is perceived as the second most regular reason of death in USA and Europe (Ruiz-Torres et al., 2017). It is claimed by the world cancer research fund international, Norway, Australia, France (metropolitan), Belgium, and Denmark were the top 5 countries with the highest cancer rates (Adjei, 2019; Tewari et al., 2019). It is said that, Asia consists about 60% of the world's population and nearly half of the world's cancer patients. The incidence of cancer is expected to rise from 6.1 million cases in 2008 to 10.6 million by 2030, due to various factors (Sankaranarayanan et al., 2014; Tewari et al., 2019). In low-income and middle-income nations, more than 80% of diagnosed instances of childhood cancer happen (Magrath et al., 2013; Rodriguez-Galindo et al., 2015; Ward et al., 2019). Despite developments in discovering new oncological therapies, cancer treatment's selectivity and effectiveness continues a major challenge (Teijeiro-Valiño et al., 2019).

1.7.3 Cancer prevalence in Bangladesh

Among the non-communicable diseases in Bangladesh, cancer in specific is one of the main causes of mortality and morbidity. Cancer is the sixth major cause of death in Bangladesh (S. A. Rahman et al., 2019). This is due to organizational healthcare system shortfalls, lack of healthcare facilities, and lack of access to facilities due to excessive therapy costs. Generally, cancer patients in Bangladesh spend an approximately $83 million per year on therapy overseas owing to the country's insufficient equipment (Hussain & Sullivan, 2013). Bangladesh has 13 to 15 lakh patients with cancer, with about 2 freshly diagnosed patients with cancer each year (Hussain & Sullivan, 2013). Pediatric cancer incidence in Bangladesh is estimated to be 13,000 instances per year and hospital therapy for fewer than 500 kids (S. A. Rahman et al., 2019).

1.7.4 Current drug in cancer treatment

Chemoprevention of cancer includes the use of a synthetic, natural or biological agent to reduce the danger or delay cancer. Several studies have revealed a reduced incidence of cancer in developing nations, particularly in population groups with fruit and vegetable-rich diets (Aqil et al., 2019). Some of our most helpful cancer drugs such as paclitaxel (Taxol), vinblastine and vincristine, irinotecan, docetaxel, topotecan, and etoposide have historically resulted from plant-derived compounds. These compounds are presently used as one of the most efficient cancer chemotherapeutics (Cragg & Pezzuto, 2016).

Table 5: Some recent anti-cancer drugs.

Drug	**Chemical class**	**Source**	**Mechanism of action**	**Reference**
Curcumin	Phenol	*Curcuma longa*	Inhibition of cell signaling pathways	(Aqil et al., 2019)
Phophyllotoxin	Resin	*Phodophyllum emodi*	Destabilizes microtubules by binding tubulin	(Ahmad et al., 2019)
Paclitaxel	Alkaloid	*Taxus brevifolia*	Increasing of tubulin polymerization	*(Tewari et al., 2019)*
Solamargines	Glycoalkaloid	*Solanum incanum* L	Up-regulation of TNF Receptors expression.	*(Tewari et al., 2019)*

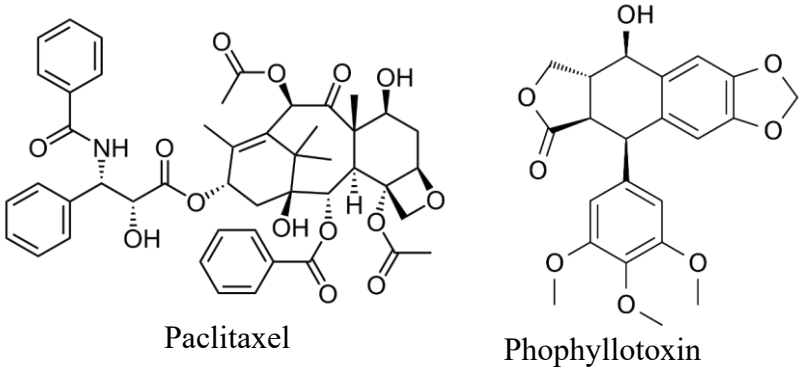


Figure 8: Chemical structure of some anti-cancer drugs.

1.7.5 *In-vitro* cytotoxicity study

In *in-vitro* toxicology research, cytotoxicity assays are commonly used. The most commonly used to detect cytotoxicity or cell viability after exposure to toxic substances are the LDH leakage assay, a protein assay, the neutral red and the MTT assay (Fotakis & Timbrell, 2006). The colorimetric MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) assay based on the reduction of yellow tetrazolium salt to violet formazan crystals by feasible cells and intracellular dehydrogenases was one of the leading lights of *in-vitro* cytotoxicity research (Damiani et al., 2019). MTT assay has a number of benefits over pre-existing cell counting and nucleotide radiolabeling techniques.

1.8 Medicinal plants in Bangladesh

1.8.1 Overview of medicinal plants in Bangladesh

Because of their positive effects, less toxicity and less expensive than modern treatments, 80% of total individuals in the globe, especially those in the urban region, are immediately relying on medicinal plants (Sarkar et al., 2019). Most of the population in Bangladesh reside in remote regions and do not use contemporary health care facilities due to inadequate road transport, the lack of allopathic physicians and hospitals or clinics and the uncompromising ability to buy contemporary medications (Dulla & Jahan, 2017). Folk medicinal practitioners (FMPs) and tribal medicinal practitioners (TMPs) expertise can serve an significant part in discovering latest drugs because they mostly use herbs in their therapy (Hosen & Rahmatullah, 2019). Some examples of commonly used medicinal plants in Bangladesh with their traditional uses are given in **Table 7** (Hosen & Rahmatullah, 2019a).

Table 6: Examples of traditionally used medicinal plants in Bangladesh.

Scientific Name	**Family Name**	**Local Name**	**Parts used**	**Ailments treated**
Calotropis procera (Ait.) Ait.f.	Apocynaceae	Akondo	Flower	Asthma, respiratory difficulties
Aloe barbadensis L.	Asphodelaceae	Ghritokumari	Leaf pulp	To brighten skin
Ocimum sanctum L.	Lamiaceae	Tulsi	Leaf	Fever, cold and coughs
Lygodium flexuosum (L.) Sw.	Lygodiaceae	Dheki shak	Leaf	Gastric problems
Azadirachta indica A. Juss.	Meliaceae	Neem	Leaf	Allergy

1.8.2 Choice of *Blumea lacera* for this project

After comprehensive study of literature about Bangladeshi medicinal plants, it was observed that methanol and water extracts of *Blumea lacera* leaf had earlier been studied using distinct cell lines for cytotoxicity. Methanol and aqueous extracts were tested against three groups of mammalian cancer cells (gastric: AGS; lung: HT-29; and breast: MDA-MB-435S) using the MTT assay for cytotoxic activity (Tiralongo et al., 2011). Methanol extract *Blumea lacera* leaf exhibited the greater cytotoxicity among all cell line samples. However, no research was observed to perform chloroform extract of *Blumea lacera* leaf for cytotoxic activity. In fact, no research was found using HeLa cell line (human cervical carcinoma) and MOLT-4 (T-cell acute lymphoblastic leukemia) cell line for cytotoxic activity of *Blumea lacera* leaf chloroform extract. Furthermore, the determination of antioxidant activity for chloroform extract of this plant leaf was not previously performed. The current study was intended to

explore antioxidant activity of *Blumea lacera* chloroform extract along with cytotoxicity activity on the HeLa and MOLT-4 cell line.

1.8.3 Description of *Blumea lacera*

Blumea lacera, also recognized in Bangladesh as Kukursunga, was an erect plant that grown throughout Bangladesh as a weed in uncultivated territories (Ibrahim et al., 2014). It was a tiny seasonal plant, camphor-like smell of leaves with yellow heads. It belonged to Asteraceae family. It was also noticed in Malaysia, India, China, tropical Africa and Australia. Various parts of the plant are traditionally used for the therapy of cholera and catarrhal diseases, as well as, an anthelmintic, diuretic, anti-scorbutic, anti-dysentric, astringent, carminative, anti-inflammatory and anti-microbial agent (Akter et al., 2016; Pratap & Parthasarathy, 2012). It has excellent dose-dependent anthelmintic activity (Pattewar et al., 2012).

Figure 9: Blumea lacera plant.

1.9 Rationale of this project

Blumea lacera had been generally used for the therapy of cholera and catarrhal diseases. It had also other benefits, such as, anthelmintic, diuretic, anti-scorbutic, anti-dysentric, astringent, carminative, anti-inflammatory and anti-microbial activity. Methanol extract of *Blumea lacera* leaf showed high cytotoxicity against three groups of mammalian cancer cells

(gastric: AGS; lung: HT-29; and breast: MDA-MB-435S). Furthermore, this plant had also been investigated for anti-thrombosis and membrane stabilizing tests that had positive results. However, chloroform extract of *Blumea lacera* leaf had not been studied before for antioxidant activity and activity for cytotoxicity in any cancer cell line. Thus, this project was performed to know about the existence of antioxidant and cytotoxic activity for chloroform extract of *Blumea lacera* leaf. This research will provide a basis for figuring out their biological and pharmacological properties and to isolate the compounds responsible for the biological properties.

1.10 Aim of this project

The aim of this project is to explore antioxidant and cytotoxic potential of *Blumea lacera* leaf extract.

1.11 Objective of this project

After investigating the literature review for *Blumea lacera* plant, the purpose of this project to use chloroform extract of *Blumea lacera* leaf was:

- Determination of antioxidant potential using multiple *in-vitro* techniques (total phenolic content (TPC), DPPH free radical scavenging assay, total flavonoid content (TFC) and total antioxidant capacity (TAC)).
- Investigation of its cytotoxicity on HeLa (human cervical cancer) and MOLT-4 (T-cell acute lymphoblastic leukemia) cell lines.

Chapter 2

Literature review

2.1 Formerly explored pharmacological functions of *Blumea lacera*

Blumea lacera literature review has been conducted and it is shown that the plant has important anti-inflammatory, analgesic, hypothermic, tranquilizing, anti-leukemic, antiviral, cytotoxic, anti-diarrheal, antimicrobial, anxiolytic, anti-atherothrombosis, cell stabilizing, alpha-amylase inhibitory, anthelmintic and astringent activity.

Table 8: Previous studies on pharmacology of Blumea lacera.

Pharmacological Activity	Type of Extracts	Plant Part	References
Anti-inflammatory	Ethanol	Aerial parts	(Basnet et al., 2015)
Anti-leukemic	Hot water	Whole plant	(Chiang et al., 2004)
Antiviral	Hot water	Whole plant	(Chiang et al., 2004)
Cytotoxic	Methanol	Leaf	(Akter et al., 2016)
Anti-diarrheal	Methanol	Leaf	(Haque et al., 2015)
Hypoglycemic	Methanol	Leaf	(Hasan et al., 2015)
Antimicrobial	Ether and methanol	Whole plant	(Jahan et al., 2014)
Anthelmintic	Ethanol and water	Whole plant	(Pattewar et al., 2012)
Anti-bacterial	Ethyl acetate and methanol	Aerial parts	(Ali Ahmed et al., 2014)
Anti-pyretic	Methanol, ethanol and chloroform	Whole plant	(Fancy et al., 2015)

2.1.1 Anti-inflammatory activity

In an earlier research, investigation of the therapeutic effectiveness of ethanol extract from *Blumea lacera* aerial parts against enterocolitis caused by indomethacin took place (Basnet et al., 2015). Male Wistar rats were split into six groups (n = 5) and administered for seven days with distinct doses of ethanolic extract (100 and 200 mg/kg) and sulphasalazine (100 mg/kg). Two subsequent doses of indomethacin (7.5 mg/kg) caused enterocolitis on the 7th and 8th day (Basnet et al., 2015). Treatments were continued and sacrificed until the 12th day. Using macroscopic results of ileum strips, modifications in biochemical parameters such as lipid peroxidation (LPO), serum lactate dehydrogenase (LDH), total thiols (TT) and tissue myeloperoxidase (MPO), the protective function was evaluated. The findings confirmed protective action of the plant against indomethacin induced enterocolitis in rats, due to its antioxidant, anti-inflammatory, antimicrobial, and membrane-stabilizing characteristics (Basnet et al., 2015).

2.1.2 Anti-leukemic activity

In an earlier research, hot water (HW) extract from the *Blumea lacera* plant had extensive anti-leukemic activity ranging from moderate to mild to L1210, Raji, anti-K562, U937 and P3HR1 leukemia cells (Chiang et al., 2004). The GI_{50} values were 175.5 ± 2.7 µg/ml, 210.4 ± 10.1 µg/ml, 216.5 ± 9.8 µg/ml and 302.2 ± 6.8 µg/ml. Against the U937 cell, GI_{50} value was > 500 µg / ml (Chiang et al., 2004).

2.1.3 Antiviral activity

In a previous study, it was observed that hot water (HW) extract from the *Blumea lacera* suppressed the replication of HSV-1 and HSV-2 viral cell line (Chiang et al., 2004). The IC_{50} results of hot water (HW) extract from the *Blumea lacera* against HSV-1 were 83.2 ± 1.5

μg/ml and 43.3 ± 25.1 μg/ml against HSV-2 (Chiang et al., 2004). As a result, it had IC_{50} values below 100 μg/ml.

2.1.4 Cytotoxic activity

Sixteen medicinal plants in Bangladesh were extracted successively with n-hexane, dichloromethane, methanol and water in a previous study. Methanol and aqueous extracts were screened three human cancer cell lines (gastric: AGS; colon: HT-29; and breast: MDA-MB-435S) using the MTT assay for cytotoxic activity (Tiralongo et al., 2011). Methanol extract of *Blumea lacera* showed the highest cytotoxicity (IC_{50} 0.01–0.08 mg/ml) against all of the cell lines tested in the study (Tiralongo et al., 2011). In another study, cytotoxic activity of *Blumea lacera* methanol extract was evaluated against two healthy cell lines (NIH3T3 and VERO) and four human cancer cell lines (AGS, HT-29, MCF-7 and MDA-MB-231) using the MTT assay for their cytotoxicity (Akter et al., 2015). It showed a considerable apoptotic potential (32% AV^+ /PI^-) on MCF-7 cells.

2.1.5 Anti-diarrheal activity

In a previous study, to assess *in-vivo* anti-diarrheal activity, methanol leaves extract of *Blumea lacera* was evaluated with *Swiss albino* mice. Compared to Loperamide, it was assessed as an antidiarrheal agent as a conventional drug. In both test cases, the 400mg/kg concentration showed a close proportion (40.275%) of Loperamide (62.068%) (Haque et al., 2015).

2.1.6 Hypoglycemic activity

A research was conducted for the preliminary exploration of hypoglycemic activity for *Blumea lacera* leaf methanol extract. The research was conducted using the oral glucose tolerance test on young male and female *Swiss albino* mice (Hasan et al., 2015). Results from that research showed that methanol leaf extract *Blumea lacera* had a dose-dependent reducing

ability of glucose at all doses (P<0.0001). Maximum hypoglycemic activity was observed as 46.85% at 400 mg/kg in glucose-induced hyperglycemic mice, while the conventional medication Glibenclamide generated 47.53% at 10 mg/kg dose (Hasan et al., 2015).

2.1.7 Antimicrobial activity

In a study, for its possible antimicrobial characteristics, the petroleum ether and methanol extract of *Blumea lacera* (whole plant) was assessed. Screening for phytochemicals of the extracts showed the presence of steroids, alkaloids, reducing sugar, tannins and gums. for the methanol extracts and petroleum ether extracts of the plant, antimicrobial activities were contrasted with that of a conventional Cefalexin antibiotic. Antimicrobial actions of the extract were screened against two bacteria and one fungus that caused multiple infectious diseases (Jahan et al., 2014). In methanol extract, petroleum ether extract and Cefalexin in Bangladesh (CFX), inhibition areas of staphylococcus aureus were 12 mm, 15 mm, and 18 mm in diameter respectively. The *Escherichia coli* inhibition area was 10 mm, 16 mm, and 20 mm, respectively, following methanol extract, petroleum ether extract and CFX (Jahan et al., 2014).

2.1.8 Anthelmintic activity

In a research, *Blumea lacera* ethanol and water extract tested against *Ascaris lumbricoides* and *Pheritema postuma* for *in-vitro* anthelmintic activity using Piperazine Citrate as standard. Study findings showed that plant extracts showed very important dose-dependent anthelmintic activity. It was observed that alcoholic extract had a slightly greater potency relative to aqueous extract (Pattewar et al., 2012b).

2.1.9 Anti-bacterial activity

The most useful part for ethno medicinal use was found in the leaf of *Blumea lacera*. A study involved comparative analysis of bacterial antibacterial activity by *Blumea lacera* from both

the Sundarbans mangrove habitat and the natural habitat of plain land (Ali Ahmed et al., 2014). In this research, 10 bacterial strains were screened to assess the widely spectated antimicrobial characteristics of the plant for the ethyl acetate extracts of the aerial components. *Bacillus subtilis* and *Bacillus cereus* are two gram positive bacteria used in the current study, as well as, 8 gram negative bacteria were used, namely *Vibrio cholerae*, *Pseudomonas* sp., *Serratia* sp., *Salmonella typhi*, *Salmonella* sp., *Proteus mirabilis*, *Erwinia* sp. and *Shigella flexneri* (Ali Ahmed et al., 2014). An inhibitory action on all tested bacteria had been shown to the ethyl acetate fraction of the sample. Inhibition area generated by the ethyl acetate fraction was 14 mm for 120 µg/ml against *Bacillus cereus*, along with 13.83 mm for 80 µg/ml and 10.67 mm for 40 µg/ml. The ethyl acetate fraction was therefore active against *Bacillus cereus*, a gram positive bacteria in a dose dependent manner (Ali Ahmed et al., 2014).

2.1.10 Anti-pyretic activity

Brewer's yeast technique had previously been used to perform an *in-vivo* antipyretic test for *Blumea lacera* leaf of methanol, ethanol and chloroform extracts (Fancy et al., 2015). The findings of the *Blumea lacera* leaf extract anti-pyretic tests using Brewer's yeast induced pyrexia in mice reveals a substantial amount of reduced pyrexia. The higher dose (400 mg/kg) of methanol and ethanol extracts (200 & 400 mg/kg) showed significant pyrexia reduction rate from high level (Fancy et al., 2015).

Chapter 3

Methodology

3.1 Collection and authentication of plant material

It was highly observed that a great number of researches have been performed and many papers have been reviewed about *Blumea lacera*. Studies showed that the methanol extract of *Blumea lacera* subjected the cancer cell line to a high cytotoxic activity. However, the chloroform extract of *Blumea lacera* leaf had not been investigated yet for *in-vitro* antioxidant activity and cytotoxic activity against HeLa (human cervical cancer) and MOLT-4 (T-cell acute lymphoblastic leukemia) cell line. The plant had been decided to be selected for inquiry by means of an extensive literature research of this plant and its accessibility. In February 2019, the entire collection was taken from Chittagong, Bangladesh. It had been presented for authentication to the Bangladesh National Herbarium (NHB), Dhaka. A week later the voucher sample was accepted and the plant (Accession No.: DACB56790) recognized and verified by the Bangladesh National Herbarium Botanist, Mirpur, Dhaka.

Table 9: Blumea lacera identification.

Plant species	Local name	Family	Plant part	Voucher no.
Blumea lacera	Kukursunga	Asteraceae	Leaves	DACB: 56790

3.2 Extraction procedure

In small-scale or small-scale research or small-scale manufacturing enterprises (SMEs), traditional techniques such as maceration and extraction are widely used. There have been significant progresses in medicinal plant processing, such as contemporary extraction techniques; microwave assisted extraction (MAE) and supercritical fluid extraction (SFE), which aim to improve yields at a reduced price. This includes developments in the processing

of medicinal plants. Furthermore, changes to methods are constantly being created. Extraction process has two parts-

a) Pre-extraction

b) Final extraction

3.2.1 Pre-extraction procedure

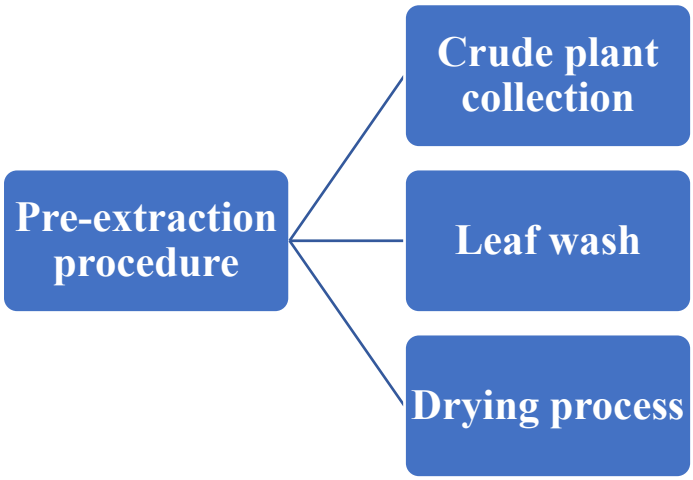


Figure 10: Pre-extraction procedure (collection, washing and drying).

The steps engaged in extracting the raw medicinal plant components. At first plant leaves were gathered and washed in order to eliminate contamination with distilled water. The leaves were then left to dry in an airtight container (approximately seven days) and the sunlight was prohibited from leafing because the sunlight might tarnish them.

3.2.2 Extraction process

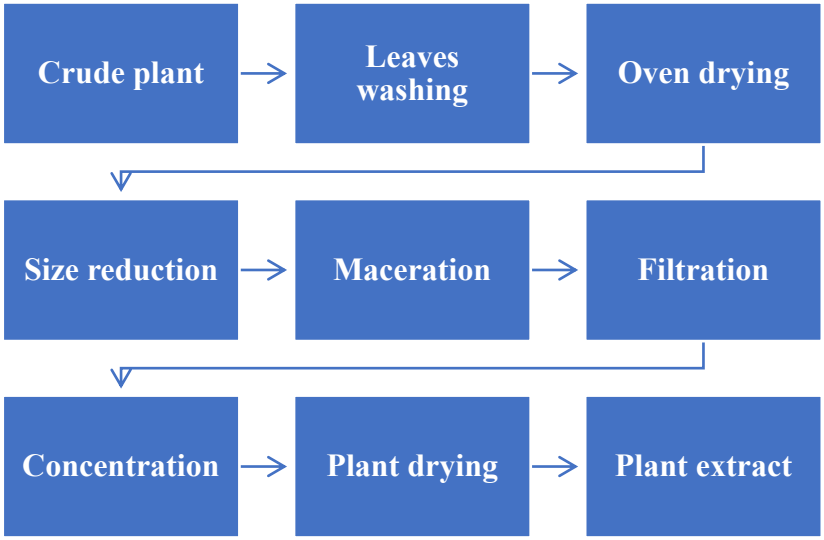


Figure 11: Schematic diagram displaying the medicinal plant extraction process.

3.2.2.1 Reduction in size and weight measurement

After leaves washing the fragile dried leaves individually ground using a high-capacity grinding machine to form coarse powder. After that packaging was lastly left in cool, dry and dark areas in airtight plastic containers with the required labelling until further examination. Necessary actions to prevent cross-contamination were taken during the grinding process. Powdered *Blumea lacera* leaf were taken and the complete weight was weighed in a weight machine using a beaker.

3.2.2.2 Extraction

This research was conducted using the maceration method for the extraction of plants and the organic solvent is chloroform. The beaker was soaked in 900 mL of chloroform for a period of seven days at normal room temperature (22 to 25°C) containing the powdered leaf. The result of the maceration was a 2-layer phase: the lowest was the sediment and the uppermost was a cloudy suspension of chloroform plant extracts.

3.2.2.3 Filtration

After maceration for a period of seven days, the contents of the beaker were filtered with Whitman filter (hole size: 110 mm) after decantation.

3.2.2.4 Concentration and drying

After collection the filtrate has been concentrated with a 100rpm (Heidolph) rotary evaporator at 30°C, until chloroform extract had been concentrated. The mixture was subsequently transferred to a dry beaker for LAF drying. In order to evaporate the solvent from the extract, the beaker was finally placed below LAF, leaving dry, semisolid extract behind. LAF was used to prevent the microbial growth of the extract while drying, recognized as a preventive measure. The beaker had been coated in aluminum foil and cooled for further use after a successful drying of the extract.

3.3 *In-vitro* antioxidant assay

Various in vitro methods are available for estimating antioxidant activities of plant extract. These four techniques were selected to estimate the antioxidant activity of the plant *Blumea lacera* chloroform extract between all the different methods: total phenol content (TPC), DPPH free radical scavenging test, total antioxidant capacity (TAC) and total flavonoid content (TFC).

3.3.1 DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay

The DPPH free radical scavenging test of chloroform extract of *Blumea lacera* was assessed using the following method (Sridhar & Charles, 2019):

3.3.1.1 Reagents and chemicals

Table 10 lists reagents and chemical substances used in determining the DPPH scavenging activity.

Table 10: Chemicals necessary to determine DPPH scavenging activity.

Name of reagent/chemical	Source
DPPH (1,1-diphenyl-2-picryl hydrazyl)	Sigma Aldrich U.S.A
Methanol	Active Fine Chemicals Ltd, Bangladesh
L ascorbic acid	Merck, Germany

3.3.1.2 Reagent preparation

2mg of DPPH was dissolved 50mL methanol to prepare 0.004% (w/v) DPPH solution that gave purple colored solution and then put in the refrigerator at 4°C till before use.

3.3.1.3 Sample and standard solution preparation

A concentration of 12mg/mL of the extract was prepared by dissolving 120mg of extract in 10mL of methanol after weighing. As a result, sample stock solution was prepared. After serial dilution of the sample stock solution, the sample concentrations were prepared to obtain 8 serially diluted concentrations: 1200, 800, 400, 200, 100, 50, 25 and 12.5 µg/mL.

L-ascorbic acid was used as standard and it was prepared similarly as the extract forming eight serially diluted concentrations, that ranged from 1200-12.5µg/mL.

3.3.1.4 Preparation of blank solution

The blank solution contained 3mL methanol.

3.3.1.5 Experimental procedure

a) Fractions of sample and standard (L-ascorbic acid) containing 1mL each were taken in test tubes.

b) 2mL of 0.004% (w/v) DPPH solution was added to each of the testubes.

c) The absorbance of the resulting solutions and control (DPPH and methanol) was measured at 517nm against blank (methanol) using a spectrophotometer (U-2910 UV-Vis Spectrophotometer) after 30 minutes of incubation at room temperature.

d) Following equation was used to calculate the percentage of free radical scavenging activity (% FRS):

$$\textbf{\% Free radical Scavenging (\%FRS) activity} = \frac{(A_0 - A_0)}{A_0} \times 100$$

Where,

A_0 = control absorbance

A_1 = sample/standard absorbance

e) Finally, IC_{50} value was measured by plotting the percent scavenging activity against concentration. To emphasize, IC_{50} value is called as the concentration at which 50% of total DPPH free radical is reduction occurred by the antioxidants.

3.3.2 Determination of total phenolic content (TPC)

The TPC of the plant extract of *Blumea lacera* was determined by the modified Folin-Ciocalteu method (Aryal et al., 2019):

3.3.2.1 Reagents and chemicals

The reagents and chemicals for the determination of total phenolic content is listed below in **Table 11**.

Table 11: Reagents and chemicals used to determine total phenolic content (TPC).

Name of reagent/chemical	Source
Folin-Ciocalteu reagent(FCR)	LOBA Chemicals Pvt. Ltd., India
Methanol	Active Fine Chemicals Ltd, Bangladesh
Gallic Acid Monohydrate	Sigma Aldrich, USA
Sodium Carbonate	Merck Specialties Pvt. Ltd., Mumbai

3.3.2.2 Reagent preparation

2mL of Folin-Ciocalteu reagent (FCR) was taken in a volumetric flask to prepare 20mL of 10% FCR solution after diluting it with distilled water.

7.5g of sodium carbonate was measured and taken in a 100mL volumetric flask to prepare 7.5% (w/v) Sodium carbonate solution after the dilution with distilled water.

3.3.2.3 Sample and standard preparation

A concentration of 12mg/mL of the extract was prepared by dissolving 120mg of extract in 10mL of methanol after weighing. As a result, sample stock solution was prepared. Four serially diluted concentrations of 1200, 800, 400 and 200 µg/mL was prepared by dissolving stock solution in methanol. The stock solution of the standard Gallic acid was prepared similarly as the extract and four serially diluted concentrations, ranging from 1200, 800, 400 and 200 µg/mL were prepared.

3.3.2.4 Preparation of the blank

The blank solution contained the same volume of sodium carbonate and FCR solution that was used in the experiment i.e. 5mL and 4mL, respectively. 1mL of methanol was used to generate up to 10 mL of the volume.

3.3.2.5 Experimental procedure

a) Fraction of sample and standard (Gallic acid) concentrations containing 1 mL was taken in test tubes.
b) 2.5mL of FCR solution was added to each testube.
c) After that, sodium carbonate solution of 2.5mL was added to each testube.
d) For a duration of 15s each of the mixture was vortexed and then heated in a water bath for 30min at 40°C.
e) Finally, using spectrophotometer (U-2910 UV-Vis Spectrophotometer), the absorbance of standard and sample solutions was measured against blank at 765nm.
f) For each of the fractions, the total phenolic content, C was measured using the following equation expressed as Gallic Acid Equivalents (GAE):

$$C = \frac{c \times V}{m}$$

Where,

C = Total phenolic content of the extract, Gallic acid (mg) per gram of dried plant extract, shown as Gallic Acid Equivalent (GAE).

c = Concentration of Gallic acid obtained from calibration curve (mg/mL)

V = Sample solution volume (mL)

m = Sample weight (g)

3.3.3 Determination of total flavonoid content (TFC)

The total flavonoid content of the extract was determined according to the following method (Pękal & Pyrzynska, 2014):

3.3.3.1 Reagents and chemicals

The reagents and chemicals for the determination of total flavonoid content are listed below in **Table 12**.

Table 12: Reagents and chemicals used to determine total flavonoid content (TFC).

Name of reagent/chemical	Source
Potassium Acetate	Merck KGAA, Germany
Methanol	Active Fine Chemicals Ltd, Bangladesh
Quercetin (standard)	Sigma Aldrich, USA
Aluminum chloride	Merck Specialties Pvt. Ltd., Mumbai

3.3.3.2 Reagent preparation

10g of aluminum chloride was measured and 10% aluminum chloride solution was prepared by diluting it with distilled water in 100mL volumetric flask making the level up to 100mL mark.

9.815g of potassium acetate was measured and 1M potassium acetate solution of 100 mL was prepared using distilled water in a 100mL volumetric flask by diluting it with up to 100mL mark.

3.3.3.3 Sample and standard preparation

Stock solution was prepared by measuring and dissolving 120mg of extract producing a concentration of 12mg/mL by adding 10mL of methanol. 4 serially diluted concentrations: 1200, 800, 400 and 200µg/mL was prepared by diluting the stock solution with methanol. For

the standard, Quercetin was used and the stock solution was prepared similarly as the extract that gave four serially diluted concentrations, that ranged from 1200, 800, 400 and 200µg/mL.

3.4.3.4 Preparation of the blank

The blank solution contained 200µL of 10% solution of aluminum chloride, 5.6mL of distilled water, 200µL of 1M potassium acetate solution and 4mL of methanol, so that the total volume of the solution became 10mL.

3.3.3.5 Experimental procedure

a) Fraction of sample and standard (Quercetin) concentrations containing 1 mL was taken in test tubes.
b) 3mL of methanol was added to each testube.
c) Then, using 1000µL micropipette, 200µL of 1M potassium acetate solution and 200µL of 10% aluminum chloride solution was added to each of the test tubes.
d) Finally, 10mL of final volume solution was prepared by adding 5.6mL of distilled water to each of the testube.
e) For a period of 30min at room temperature, the test tubes were then incubated.
f) Afterwards, using spectrophotometer (U-2910 UV-Vis Spectrophotometer), the absorbance of each of the sample and standard solutions were measured at 415nm against blank.
g) For each of the fractions, the total flavonoid content, C was measured using the following equation expressed as Quercetin Equivalents (QE):

$$C = \frac{c \times V}{m}$$

Where,

C = Total flavonoid content of the extract, Quercetin (mg) per gram of dried plant extract, shown as quercetin equivalents (QE)

c = Concentration of Gallic acid obtained from calibration curve (mg/mL)

V = Sample solution volume (mL)

m = Sample weight (g)

3.3.4 Determination of total antioxidant capacity (TAC)

The total Antioxidant capacity of plant extract of *Blumea lacera* was determined by using the following method (SC, Beldal, & Londonkar, 2019):

3.3.4.1 Reagents and chemicals

Table 13 lists below reagents and chemicals used to establish the total antioxidant capacity.

Table 13: Reagents and chemicals used to determine total antioxidant capacity (TAC).

Name of reagent/chemical	Source
Ammonium Molybdate	Active Fine Chemicals Ltd, Bangladesh
Methanol	Active Fine Chemicals Ltd, Bangladesh
L-ascorbic acid	Merck, Germany
Trisodium Phosphate ($Na_3PO_4.12H_2O$)	Merck, Germany
Concentrated Sulfuric Acid	Merck, Germany

3.3.4.2 Reagent preparation

3.28mL of 98% concentrated sulfuric acid was measured and taken in 100mL volumetric flask to prepare 100mL of 0.6M Sulfuric acid after diluting it with distilled water up to 100mL mark.

4.494g of ammonium molybdate was measured and taken in a 100mL volumetric flask to prepare 100mL of 0.004M ammonium molybdate solution after diluting it with distilled water up to 100mL mark.

1.0645g of trisodium phosphate was measured and taken in a 100mL volumetric flask to prepare 100mL of 0.028M trisodium phosphate solution after diluting it with distilled water to 100mL mark.

3.3.4.3 Sample and standard preparation

Stock solution was prepared by measuring and dissolving 120mg of extract producing a concentration of 12mg/mL by adding 10mL of methanol. 4 serially diluted concentrations: 1200, 800, 400 and 200µg/mL was prepared by diluting the stock solution with methanol. As the standard Ascorbic acid was used and the stock solution was prepared similarly as the extract with four serially diluted concentrations, that ranged from 1200, 800, 400 and 200µg/mL.

3.3.4.4 Preparation of the blank

3mL of reagent solution and 300µL of methanol was used for preparing the blank solution.

3.3.4.5 Experimental procedure

a) Fractions of sample and standard (L-ascorbic acid) of 300µL concentrations were taken in test tubes.
b) After that, test tubes were filled with 3mL of each reagent solution (0.6M sulfuric acid, 0.028M sodium phosphate and 0.004M ammonium molybdate).
c) For a period of 90min, incubation of the test tubes (sample, standard and blank) were done at 95°C in a water bath.
d) Finally, using spectrophotometer (U-2910 UV-Vis Spectrophotometer), the absorbance of the sample and standard solutions was taken against blank at 695nm.

e) For each of the fractions, the total antioxidant capacity, C was measured using the following equation expressed as Ascorbic Acid Equivalents (AAE):

$$C = \frac{c \times V}{m}$$

Where,

C = Total antioxidant capacity, Ascorbic acid (mg) per gram of dried plant extract, shown as Ascorbic Acid Equivalent (AAE)

c = Concentration of ascorbic acid obtained from calibration curve (mg/ mL)

V = Sample solution volume (mL)

m = Sample weight (g)

3.4 Cytotoxic activity study by MTT assay

3.4.1 HeLa cell line (human cervical cancer)

Organism: *Homo sapiens* (human)

Tissue: Cervix

Morphology: Epithelial

Cell type: Epithelial

3.4.1.1 Description

HeLa cells are human cervical cancer cells. Cells of the cervical human cervix are cells of the human cervix. Looking at the chromosomes, the distinction between ordinary and HeLa cells become most apparent. HeLa cells are fast and easy to grow. Throughout the past half a century, HeLa cells have become the most commonly utilized human cell line for biological studies. Cells of HeLa cell line was taken from the Centre for Advanced Research in Sciences

(CARS). This cell line was cultivated and preserved using cell culture flask in DMEM (Dulbecco's Modified Eagles Medium). It was stored in nitrogen fluid. DMEM is widely accepted and commonly used medium for various cell lines. Glucose, vitamins and amino acids are highly used to assist cells get the food they need.

3.4.2 MOLT-4 cell line

Organism: *Homo sapiens* (human)

Tissue: Blood

Morphology: Lymphoblast

Cell type: T lymphoblast

3.4.2.1 Description

Cell line MOLT-4 is a T cell line derived from a patient's leukaemia cells in a relapse who was suffering acute lymphoblastic leukaemia. In the Dulbecco Modified Eagle medium (DMEM) this cell line was cultivated by the use of cell culture flask. Human MOLT-4 cells were produced with a pair of millimetre L-glutamine in the medium of RPMI 1640 and log section was utilized for growth. A follow-up heat dehydration method was used to confirm the attachment of MOLT-4 cells and also the stability of cell proteins.

3.4.3 Solutions preparation

3.4.3.1 1% Penicillin-Streptomycin solution

The MTT test generally uses penicillin- streptomycin solution known as pen-strep to manage bacterial contamination and retain the sterilized condition throughout the procedure. The solution contained a blend of 10000 units of 10 mg of streptomycin per mL and penicillin per mL. In case of MOLT-4 line, RPMI containing 1% penicillin-streptomycin was used where RPMI 1, also known as the RPMI medium, is a cell culture growth medium. DMEM

(Dulbecco's Modified Eagle Medium) is the most suitable medium for the culture of cells and tissues for many adherent cell phenotypes among defined media. The modification of Dulbecco is an enhanced additional formulation that boosts by up to fourfold the selection of amino acid and vitamin content of the original medium of Eagle.

3.4.3.2 10% Fetal bovine serum

To prepare 10% of fetal bovine serum (FBS), 500 mL of (FBS) had been added to the 500 mL of DMEM. FBS contains a high level of albumin that offers a nutrient and atmosphere for the rapid growth of cells.

3.4.3.3 2% DMSO solution

To use as a negative control, 60µl DMSO was added to prepare 2% DMSO solution by adding in 2940 mL of distilled water.

3.4.3.4 Trypsin

In the medium 0.25% trypsin was used.

3.4.3.5 Used consumables

96-well plate, 15-mL tubes, Tips, Gloves, PTFE Syringe Filter (0.45µm pore size and 25mm diameter), Culture flask, Cell culture media, 1% Penicillin-Streptomycin, Gentamycin, Serological pipette, Trypsin etc.

3.4.3.6 Used instruments

Haemocytometer, Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA), trinocular Microscope with Camera (Olympus, Japan), CO_2 incubator (Nuaire, USA).

3.4.3.7 Celltiter 96 assay kit

The Celltiter 96 assay kit consists of a combination of skilled reagents allowing proliferation and cytotoxicity to be rapidly established as an advantageous model. The aim is to change the

cell's division into a formazan that is definitely distinguished with a 96-plate using tetrazolium salt. For this experiment in 96 well plates in cell culture, a colour solution mixer was introduced.

3.4.4 Preparation of the different concentrations of plant extract

The test was conducted at 4 concentrations: 2.5mg/mL, 0.25mg/mL, 0.025mg/mL and 0.0025 mg/mL of the *Blumea lacera* leaves extract of choloroform. Stock solution of 20 mg/mL concentration was made by measuring and adding 20 mg of the extract in 1mL DMSO solution. After diluting the stock solution 2.5 mg/mL solution was prepared and then it was further diluted to prepare 0.25 mg/mL. 0.25 mg/mL solution was diluted to prepare 0.025 mg/mL concentration by DMSO. In this manner, by serial dilution with DMSO, 0.0025 mg/mL concentration was made. Before examination, the samples were filtered through a 0.45µm syringe filter.

3.4.5 Cell culture

3.4.5.1 Preparation of assay plates

DMEM (Dulbecco's Modified Eagle's Medium) and RPMI were used for the maintenance of HeLa and MOLT-4 cell lines respectively. 10% fetal bovine serum, 0.2% Gentamicin and 1% penicillin-streptomycin were added on each cell line.

3.4.5.2 Thawing of cells

MOLT-4 and HeLa cells had been preserved in cryovials containing liquid nitrogen. By swirling the vial delicately using a water bath at 37 ° C, the cryovial was taken and quickly defrosted until a small piece of ice was left in the vial. The thawed HeLa and MOLT-4 cells were subsequently transferred to two centrifuge tubes containing the DMEM medium and RPMI medium under a laminar airflow hood with drop wise. The cells were then gently suspended in the medium and transferred to the vessels of culture.

3.4.5.3 Cell passage

Cell passage was done by transferring the cells into a new medium to obtain a fresh cell suspension. FBS washed the used cultured media and then added 800µl of trypsin to detach the cells from the top of the culture vessels. Then the cells were incubated and microscopically checked for detachment. After 90 percent of cells were watched detached, 5 mL of DMEM media was added to the vessel comprising HeLa cells and 5 mL of RPMI media was added to the vessel comprising MOLT-4 cells and mixed with a pipette. Lastly, 1 mL of these solutions was drawn and blended in fresh vessels with 4 mL of DMEM and 4 mL of RPMI combined and retained for further use in an incubator.

3.4.5.4 Harvesting of cell

In the growth of the log phase, the cell was collected using trypsin. Then cell was counted in 96 well plates and seeded.

3.4.5.5 Counting of cells

Using a haemocytometer, cell counting had been done. The haemocytometer was prepared with ethanol and lens paper by deliberately cleaning and polishing the mirror like surface. The coverslip was put on the surface of the counting. Using a Pasteur pipette, the fresh cell suspension was introduced into the haemocytometer. Sufficient suspension was presented to simply overflow the surface. Then in the microscope stage, the counting chamber was set, then the counting grid was focused. At 40X magnification, 1 whole grid can be observed in a standard haemocytometer with rulings. The 4 large squares ' cells have been counted. Either the upper and left sides were counted to touch cells or the lower or right sides to touch cells.

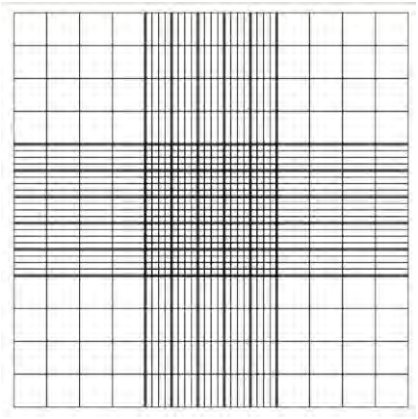


Figure 12: Haemocytometer.

3.4.6 Procedure

The cytotoxic effect was carried out using their services at the Centre for Advanced Research in the Sciences (CARS). The MTT colorimetric test was conducted using celltiter 96 non-radioactive cell proliferation assay kit (Promega, USA). At 37°C and 5% carbon dioxide environment, cells were placed on 96 well plates and incubated. 10µL of test was introduced to each well after 24 hours of incubation. Then for 2 days it was incubated again. Cytotoxicity was examined using celltiter 96 non-radioactive cell proliferation assay kit after 2 days of incubation. Using a 96-well plate reader, the absorbance was then measured at 570 nm. For all 5 concentrations and negative control, the same procedure had been followed. With 2 percent DMSO solution, negative control contained in medium and blank was contained only medium. For each sample, duplicate wells were used.

Cytotoxic activity was calculated by using a formula which is given below-

$$\% \text{ of cytotoxic activity} = \left(100 - \frac{Absorbance\ of\ test\ sample}{Absorbance\ of\ negative\ control}\right) \times 100$$

Chapter 4

Results

4.1 Calculation of percent yield of the plant extract *Blumea lacera*

4.1.1 Net weight of the plant extract obtained after maceration process

The maceration technique reduced plant powder and was used for further tests.

Table 14: Total weight of the chloroform leaf extract of the plant.

Weight of plant leaf powder	132.92 g
Initial weight of the petri dish	103.29 g
Weight of the petri dish and extract	119.04g
Final weight of extract	15.75g

Interpretation: After successive extraction and drying method, 15.75 g of chloroform leaf extract of *Blumea lacera* was obtained from 132.92 g of plant leaf powder. This extract was used for different experiments.

4.1.2 Calculation of the percent yield of chloroform leaf extract

Percent yield (%) = $(W_1 \times 100) / W_2$

Where, W_1 = Net weight of extract after maceration in gram

W_2 = Total weight of powder taken for extraction in gram

So,

Percent yield (%) = $(W_1 \times 100) / W_2$

$= (15.75 \times 100) / 132.92$

$= 20.94$ %

Interpretation: Following the maceration process, the percentage yield of leaf extract was found to be 20.94%.

4.2 DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay

4.2.1 Table of DPPH free radical scavenging assay

The concentration and absorbance values of the sample (*Blumea lacera*, BL) and standard (Ascorbic acid, AA) are shown in the **Table 15**.

Table 15: DPPH free radical scavenging assay (Absorbance vs. Concentration).

Concentration (µg/mL)	**Absorbance of Standard (AA) at 517nm (Mean ± SDV)**	**Absorbance of Sample (BL) at 517nm (Mean ± SDV)**
12.5	0.18 ± 0.001	0.53 ± 0.005
25	0.08 ± 0.001	0.53 ± 0.001
50	0.06 ± 0.006	0.52 ± 0.005
100	0.06 ± 0.002	0.47 ± 0.007
200	0.05 ± 0.001	0.37 ± 0.041
400	0.05 ± 0.003	0.24 ± 0.058
800	0.05 ± 0.006	0.12 ± 0.001
1200	0.04 ± 0.004	0.11 ± 0.079
The data was presented as mean ± standard deviation of duplicate experiments.		

Interpretation: Corresponding to their concentration, absorbance values and the mean of the sample at 517 nm are indicated in **Table 15**. The concentrations are ranging from 12.5µg/mL to 1200µg/mL. From this table it was observed that the absorbance for both of the standard and sample were decreasing with the increase of concentration. However, between the concentration 50µg/mL to 1200µg/mL, not much difference in absorbance was seen for

standard. On the other hand, between the concentration 50µg/mL to 1200µg/mL, absorbance decreased slowly for the sample.

4.4.2 Graph of Absorbance vs. Concentration for BL and AA in DPPH free radical scavenging assay.

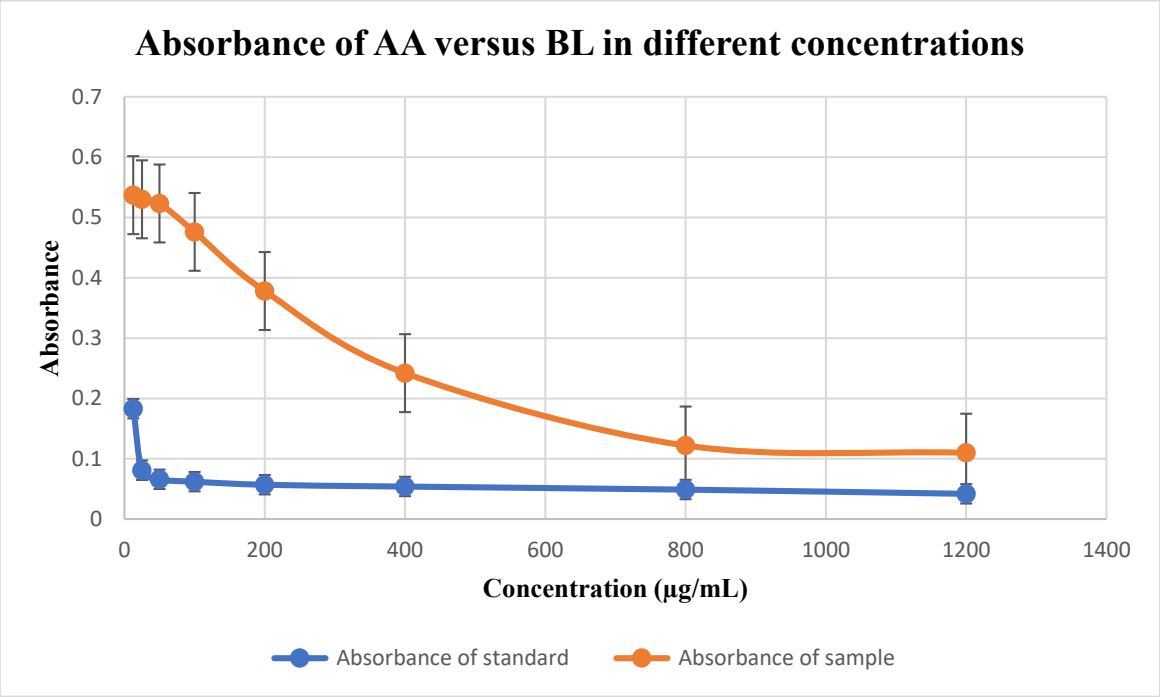


Figure 13: Graph of Absorbance vs. Concentration (µg/mL) for AA and BL in DPPH free radical scavenging assay.

Interpretation: From the **Table 15** and **Figure 13**, it was clearly observed that concentration (from 12.5µg/mL to 1200µg/mL) of *Blumea lacera* was inversely proportional to that of absorbance. For example, when the concentration increased gradually from 12.5µg/mL to 1200µg/mL, the absorbance values decreased slowly from 0.53 to 0.11. On the other hand, in case of Ascorbic acid, the absorbance decreased slowly from 0.18 to 0.04; thus, implying that the sample (red) showed flatter slope with a lower inhibition of DPPH free radicals than the standard Ascorbic acid showing steeper slope (blue).

4.2.3 Table of percentage (%) of inhibition of AA and BL

The **Table 16** shows the percentage of inhibition of DPPH free radicals by chloroform extract of *Blumea lacera* leaf.

Table 16: Inhibition of DPPH free radicals by chloroform leaf extract of BL with respect to standard AA.

Concentration (µg/mL)	**% of Inhibition (AA) (Mean ± SDV)**	**% of Inhibition (BL) (Mean ± SDV)**
12.5	77.43 ± 0.123	33.78 ± 0.652
25	90.01 ± 0.123	34.64 ± 0.213
50	91.86 ± 0.739	35.51 ± 0.616
100	92.35 ± 0.246	41.30 ± 0.863
200	92.97 ± 0.123	53.39 ± 5.064
400	93.34 ± 0.369	70.16 ± 7.233
800	93.95 ± 0.739	84.95 ± 0.123
1200	94.82 ± 0.493	86.43 ± 9.787
Data was presented as mean ± standard deviation for the experiments.		

Interpretation: From the **Table 16**, it was noticed that % inhibition of DPPH free radical scavenging activity gradually increased corresponding to their concentration (12.5µg/mL to 1200µg/mL) in both sample (BL) and standard (AA). However, it was found that percent (%) inhibition of DPPH free radical scavenging activity by chloroform extract of *Blumea lacera* leaf was lower than ascorbic acid in correspondence to their concentration. The highest DPPH free radical scavenging activity was found to be at 1200µg/mL concentration for both sample and standard. For sample, it was 86.43% and for standard, it was 94.82%.

4.2.4 Graph of percent (%) of inhibition versus concentration by AA and BL

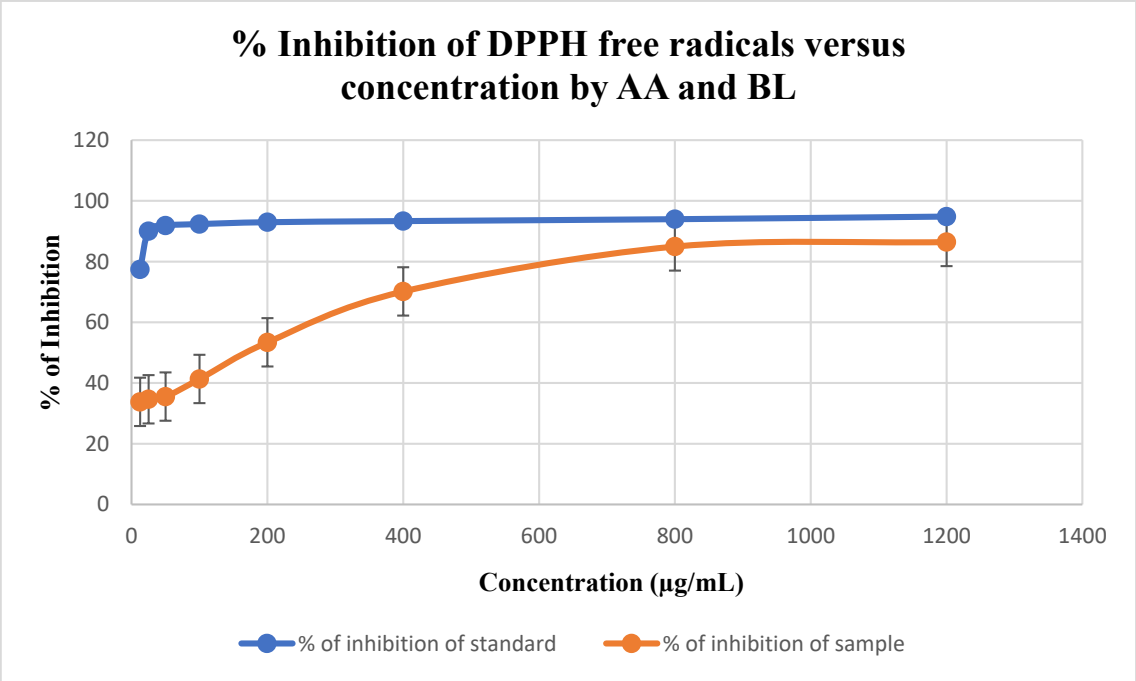


Figure 14: Percent inhibition of DPPH free radical scavengers by AA and BL versus concentration.

Interpretation: From the **Figure 14**, we can observe that % of inhibition was increasing with their respective concentrations in case of chloroform extract of *Blumea lacera* leaf. However, it was slightly lower than % of inhibition of ascorbic acid. In addition, the alleviation in % of inhibitory activity of BL was not in a constant manner. Furthermore, for both sample and standard, the highest free radical inhibition was found at 1200µg/mL.

4.2.5 IC_{50} value determination of ascorbic acid and chloroform extract of *Blumea lacera*

Table 17: IC_{50} values of sample and standard.

Sample *(Blumea lacera)*	2.39 µg/mL
Standard (Ascorbic acid)	1.32 µg/mL

Interpretation: The IC_{50} value of chloroform extract of *Blumea lacera* leaf showed that for the inhibition of 50% of all DPPH free radicals, low concentration (2.39 µg/mL) was required. Also, low concentration (1.324 µg/mL) was required to achieve the same inhibitory effect for Ascorbic acid. Thus, the IC_{50} value of the sample was comparable to that of standard. Therefore, the **Table 17** showed that the plant extract used for this study had exhibited high antioxidant potential.

4.3 Determination of total phenolic content (TPC)

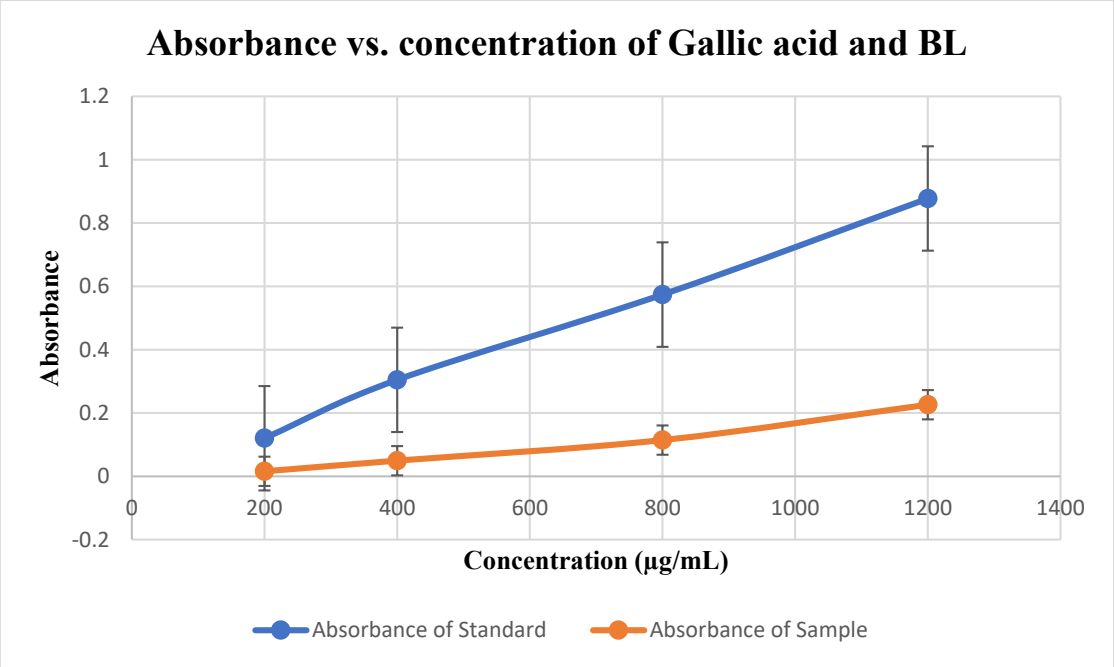


Figure 15: Graph of Absorbance vs. Concentration (µg/mL) for Gallic acid and BL.

Interpretation: From the **Figure 15** it was observed that, with the increase in concentration, the absorbance is increasing for the standard in a significant manner. On the other hand, the absorbance of the sample is also increasing with the concentration but not significantly.

4.3.1 Calibration curve of Gallic acid

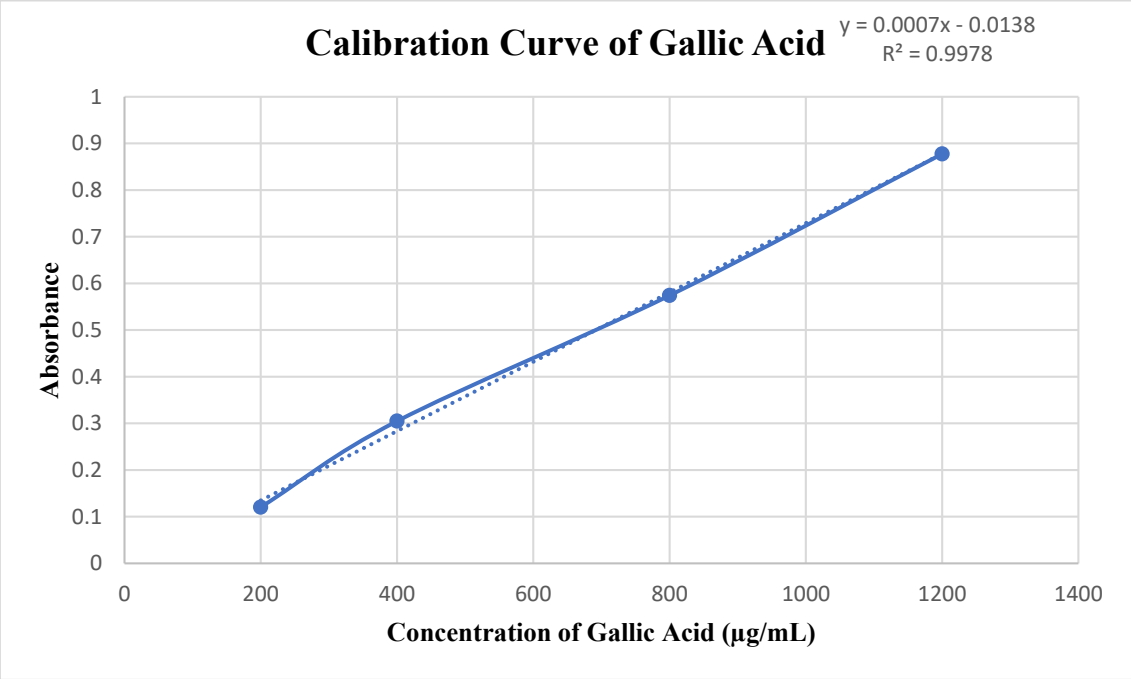


Figure 16: Standard curve of Gallic acid to determine the TPC in sample.

Interpretation: Figure 16 showed that with the increase of concentration of the standard (Gallic acid), the absorbance was also increasing. The highest absorbance was observed at 1200µg/mL.

4.3.2 Table of total phenolic content (TPC) in *Blumea lacera* leaf

Table 18: Total phenolic content (TPC) of Blumea lacera (BL) as Gallic acid equivalent (GAE).

Concentration of sample (µg/mL)	TPC (GAE) (Mean ± SDV)
200	210.47 ± 22.961
400	225.47 ± 5.455
800	228.80 ± 7.216
1200	285.47 ± 3.149
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It was observed from **Table 18** and **Figure 16** that, TPC of *Blumea lacera* is concentration dependent. With the increase in concentration (200µg/mL to 1200µg/mL) of chloroform extract of *Blumea lacera* leaf, total phenolic content also increased from 210. 47 mg to 285.47 mg as GAE per gram of dried extract. It was known that the antioxidant activity increases with the increase of the total phenolic content. The extract showed highest amount phenolic content at the concentration of 1200µg/mL, which was 285.47mg as GAE per gram of dried extract. As a result, chloroform extract of the leaf showed high antioxidant potential.

4.4 Determination of total flavonoid content (TFC)

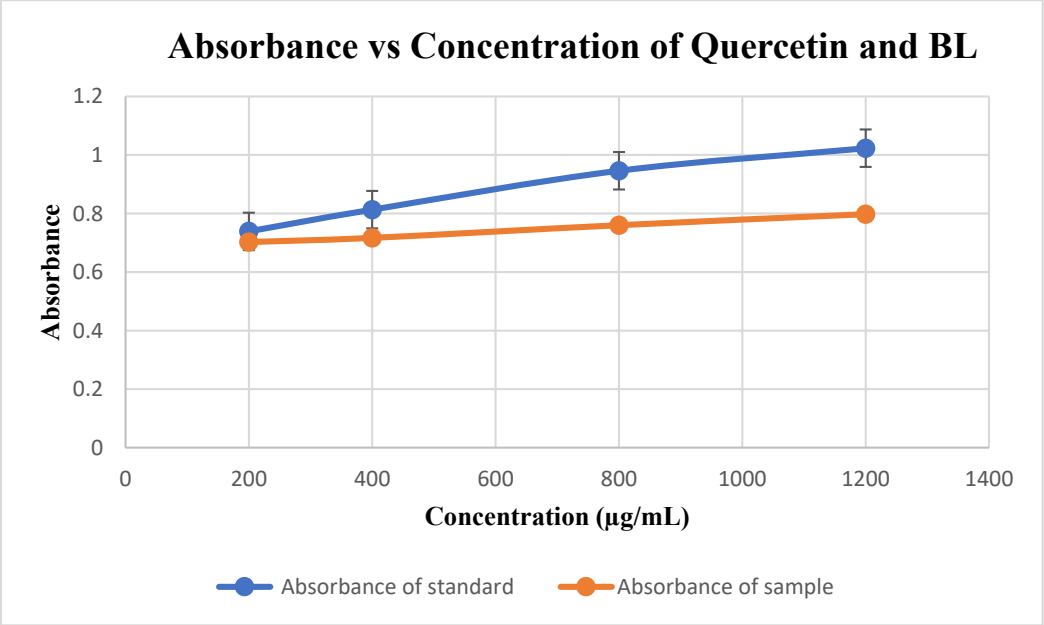


Figure 17: Graph of Absorbance vs. Concentration (µg/mL) for Quercetin and BL.

Interpretation: From the **Figure 17** it was observed that, with the increase in concentration, the absorbance is increasing for the standard in a significant manner. On the other hand, the absorbance of the sample is also increasing with the concentration but not significantly.

4.4.1 Calibration curve of Quercetin

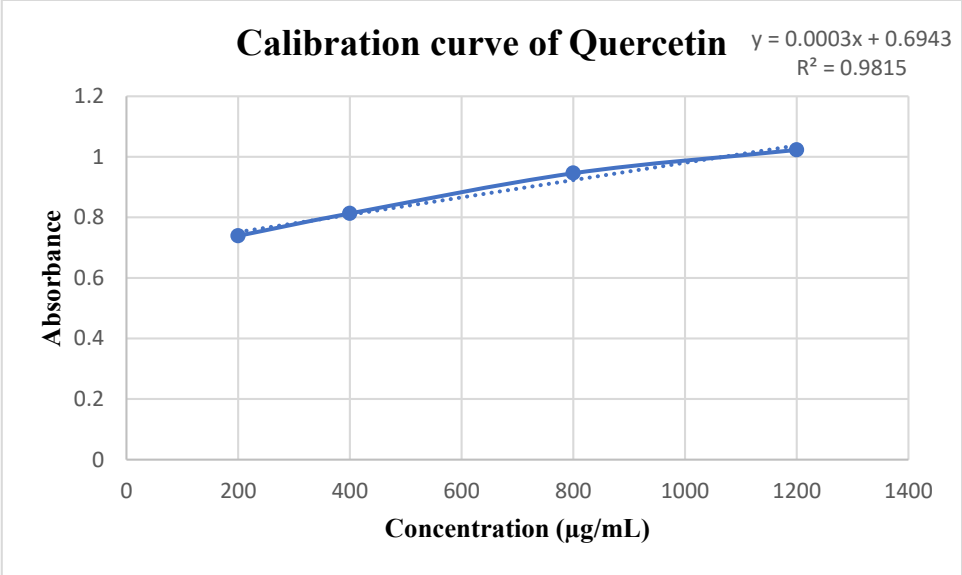


Figure 18: Standard curve of Quercetin to determine the TFC in sample.

Interpretation: Figure 18 showed that with the increase of concentration of the standard (Quercetin), the absorbance was also increasing. The highest absorbance was observed at 1200µg/mL.

4.4.2 Table of total flavonoid content (TFC) in *Blumea lacera* leaf

Table 19: Total flavonoid content (TFC) of Blumea lacera (BL) as Quercetin equivalent (QE).

Concentration of sample (µg/mL)	TFC (QE) (Mean ± SDV)
200	133.88 ± 25.458
400	186.38 ± 12.729
800	273.75 ± 12.500
1200	287.12 ± 4.243
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It was noticed from **Table 19** and **Figure 18** that, TFC of *Blumea lacera* was concentration dependent. With the increase in concentration (200µg/mL to 1200µg/mL) of

chloroform extract of *Blumea lacera* leaf, total flavonoid content also increased from 133.88 mg to 287.12mg as QE per gram of dried extract. It was known that the antioxidant activity increases with the increase of the total flavonoid content. The extract showed highest amount flavonoid content at the concentration of 1200µg/mL, which is 287.12mg as QE per gram of dried extract. As a result, chloroform extract of the leaf showed high antioxidant potential.

4.5 Determination of total antioxidant capacity (TAC)

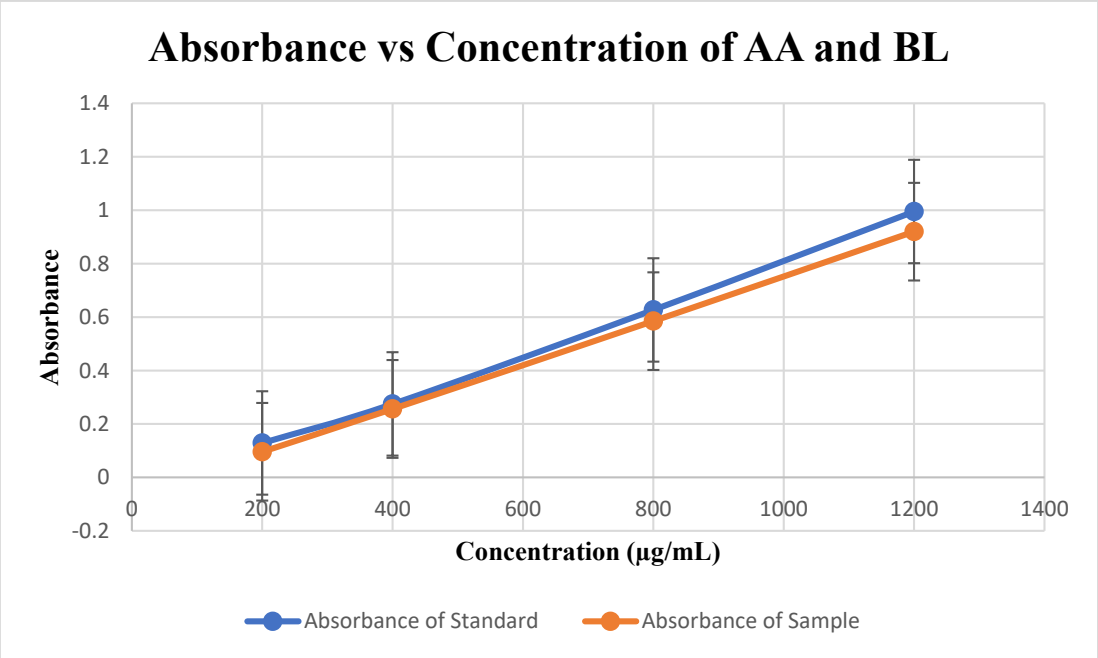


Figure 19: Graph of Absorbance vs. Concentration (µg/mL) for Ascorbic acid and BL.

Interpretation: From the **Figure 19** it was observed that, with the increase in concentration, the absorbance is increasing for the standard in a significant manner. On the other hand, the absorbance of the sample is also increasing with the concentration but not significantly.

4.5.1 Calibration curve of ascorbic acid (AA)

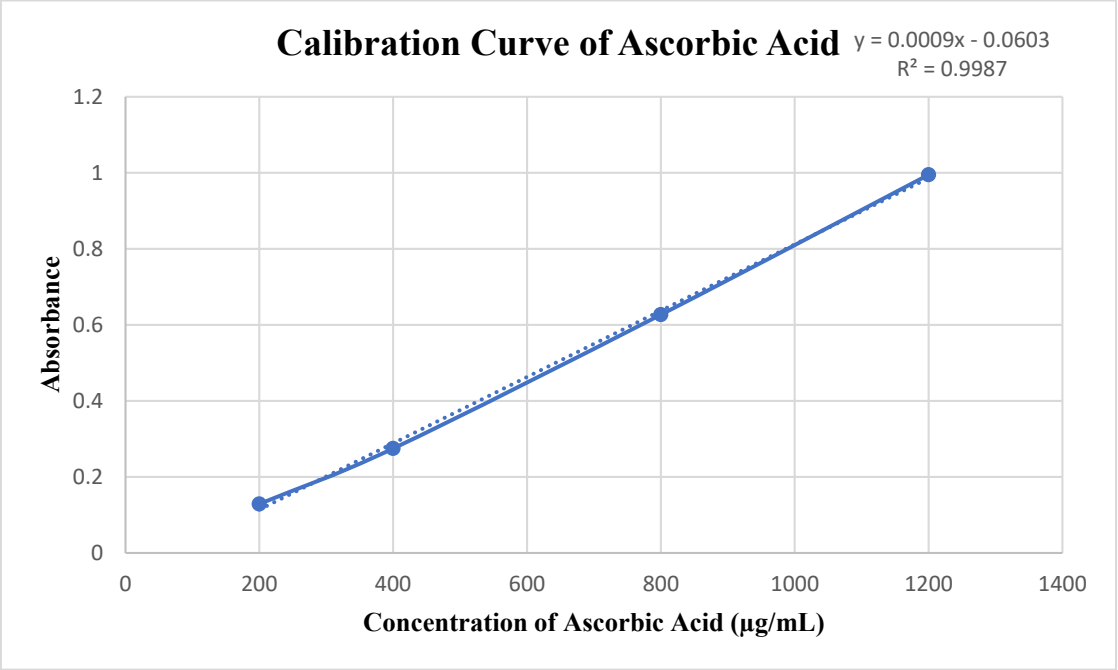


Figure 20: Standard curve of Ascorbic acid to determine the TAC in sample.

Interpretation: Figure 20 showed that with the increase of concentration of the standard (Ascorbic acid), the absorbance was also increasing. The highest absorbance was observed at 1200µg/mL.

4.5.2 Table of total antioxidant capacity (TAC) in *Blumea lacera* leaf

Table 20: Total antioxidant capacity (TAC) of Blumea lacera (BL) as Ascorbic acid equivalent (AAE).

Concentration of sample (µg/mL)	TAC (AAE) (Mean ± SDV)
200	260.50 ± 2.866
400	263.86 ± 1.734
800	268.87 ± 1.909
1200	272.21 ± 0.848
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It was observed from **Table 20** and **Figure 20** that, TAC of *Blumea lacera* is concentration dependent. With the increase in concentration (200µg/mL to 1200µg/mL) of chloroform extract of *Blumea lacera* leaf, total antioxidant capacity also increased from 260.50mg to 272.21mg as AAE per gram of dried extract. However, the alleviation in antioxidant capacity had not occurred constantly. It was known that the antioxidant activity increases with the increase of the total antioxidant capacity. The extract showed highest amount antioxidant activity at the concentration of 1200µg/mL, which is 272.21mg as AAE per gram of dried extract. As a result, chloroform extract of the leaf showed high antioxidant potential.

4.6 *In-vitro* cytotoxic activity of chloroform extract of *Blumea lacera* leaf on HeLa and MOLT-4 cell line by MTT assay

The cytotoxicity activity of chloroform extract of *Blumea lacera* was accomplished by MTT assay on HeLa and MOLT-4 cell line. Different concentrations (0.0025mg/mL, 0.025mg/mL, 0.25mg/mL and 2.5mg/mL) of the chloroform extract of the sample were used to investigate the cytotoxic activity. 2% DMSO in DMEM medium was used as a negative control. Absorbance was observed for each of the concentrations.

4.6.1 HeLa cell line

Survival of cells and % of cell growth inhibition in different concentrations along with their IC_{50} value for HeLa cell line are given in the **Table 21** and images are attached in **Figure 21**.

Table 21: Survival and inhibition of HeLa cells in different concentrations along with their IC_{50} value.

Sample concentration	**% of cell growth inhibition**	**IC_{50} (mg/mL)**
2% DMSO (negative control)	0	3.46
0.0025mg/mL	3.47	
0.025mg/mL	17.18	
0.25mg/mL	27.26	
2.5mg/mL	39.17	

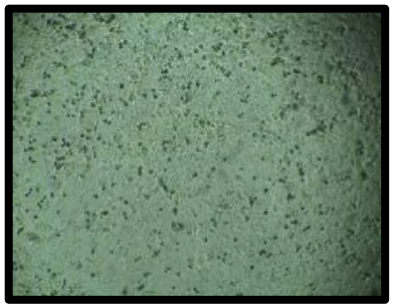
Interpretation: According to the percentage of the survival of the HeLa cells that was observed through the trinocular microscope, it is evident that highest cytotoxicity observed on HeLa cell line at 2.5 mg/mL and low cytotoxicity observed at 0.0025 mg/mL concentration of sample extract. When the concentration of sample extract was highest (2.5mg/mL), highest cell death was detected (39.17% cell death) after 48 hours of incubation. Extracts exhibited low cytotoxic activity at the other three concentrations (0.0025mg/mL, 0.025mg/mL and 0.25mg/mL) as the percentage of inhibition was 3.47%, 17.18% and 27.26%, respectively. The IC_{50} value was found to be 3.46mg/mL which meant 3.46mg of plant extract was needed to inhibit 50% of cell growth. As a result, it showed low cytotoxic activity.

(a) 2% DMSO as negative control

(b) 0.0025 mg/mL of plant extract

(c) 0.025 mg/mL of plant extract

(d) 0.25 mg/mL of plant extract



(e) 2.5 mg/mL of plant extract

Figure 21 (a), (b), (c), (d), (e): HeLa cell viability chloroform extract of Blumea lacera leaf at different extract concentration.

Interpretation: Figure 21 showed the cell viability of HeLa cells in different concentrations of the extract as well as in the negative control (2% DMSO). In the figure **21(a)** depicted the negative control where only 2% DMSO solution was added. In the Figure **21(b)**, it was seen that the cell viability decreased due to adding leaf extract of 0.0025mg/mL concentration; 3.47% cells growth inhibition was observed. The **Figure 21(c)** showed slightly less HeLa cell viability in comparison to second picture as leaf extract of 0.025mg/mL concentration was added. In the fourth picture i.e. **Figure 21(d)** 0.25mg/mL concentration of extract was added and a visible cell growth inhibition was observed which was approximately 27.26%. The **Figure 21(e)** showed cell viability at 2.5mg/mL plant extract concentration which showed

highest cytotoxicity on HeLa cell line. Lastly, **Figure 21(e)** depicted the 2.5mg/mL concentration of extract killing the maximum number of cells and exhibiting low cytotoxicity with almost 39.17% of cell growth inhibition as the IC_{50} value was found to be 3.46mg/mL.

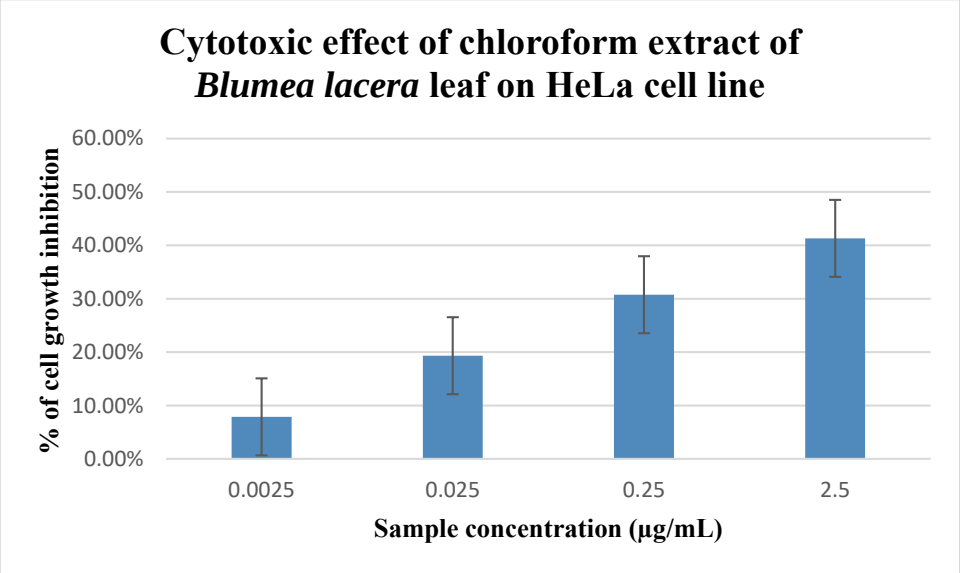


Figure 22: Cytotoxic effect of chloroform extract of Blema lacera leaf on HeLa cell line.

Interpretation: Figure 22 was the graphical representation of the percentage of cell growth inhibition versus the respective concentrations of plant extract. From the bar chart representation, it was very much evident that the lowest percentage of cell growth inhibition was observed at 0.0025mg/mL concentration of the plant extract depicting a greater cell viability. On the other hand, the lowest cell viability and the greatest percentage of cell growth inhibition was observed at 2.5mg/mL concentration of plant extract.

4.6.2 MOLT-4 cell line

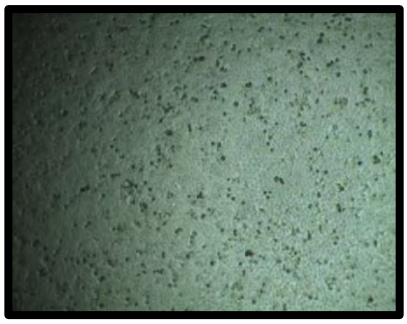
Survival of cells and % of cell growth inhibition in different concentrations along with their IC_{50} value for MOLT-4 cell line are given in the **Table 22** and images are attached in **Figure 23**.

Table 22: Survival and inhibition of MOLT-4 cells in different concentrations along with their IC_{50} value.

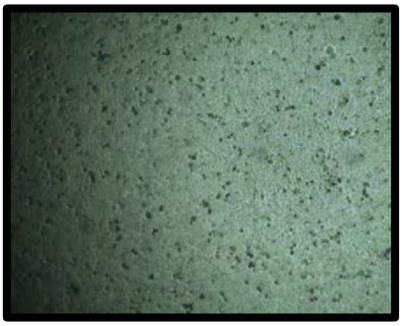
Sample concentration	**% of cell growth inhibition**	**IC_{50} (mg/mL)**
2% DMSO (negative control)	0	3.29
0.0025mg/mL	7.89	
0.025mg/mL	19.34	
0.25mg/mL	30.76	
2.5mg/mL	41.29	

Interpretation: According to the percentage of the survival of the MOLT-4 cell that was observed through the trinocular microscope, it is evident that highest cell toxicity observed on MOLT-4 cell line at 2.5mg/mL and low cytotoxicity observed at 0.0025mg/mL concentration of sample extract. When the concentration of sample extract was highest (2.5mg/mL), highest cell death was detected (41.29% cell death with 68.56% viable MOLT-4 cells) after 48 hours of incubation. Extracts exhibited low cytotoxic activity at the other three concentrations (0.0025mg/mL, 0.025mg/mL and 0.25mg/mL) as the percentage of inhibition was 7.89%, 19.34% and 30.76%, respectively. The IC_{50} value was found to be 3.29mg/mL which meant 3.29mg of plant extract was needed to inhibit 50% of cell growth. As a result, it showed low cytotoxic activity.

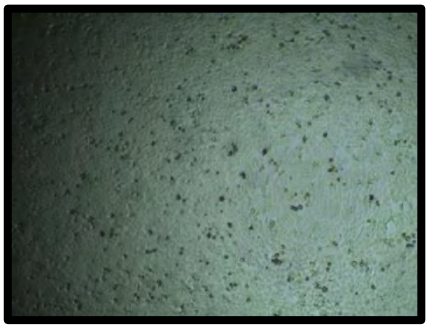
(a) 2% DMSO as negative control



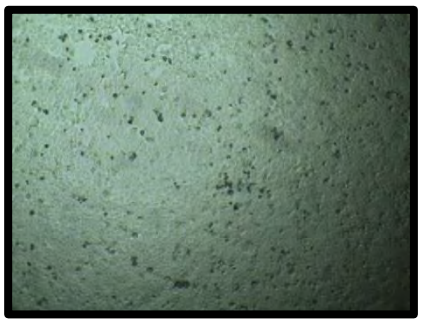
(b) 0.0025 mg/mL of plant extract



(c) 0.025 mg/mL of plant extract



(d) 0.25 mg/mL of plant extract



(e) 2.5 mg/mL of plant extract

Figure 23 (a), (b), (c), (d), (e): MOLT-4 cell viability of chloroform extract of Blumea lacera leaf at different extract concentration.

Interpretation: Figure 23 showed the cell viability of MOLT-4 cells in different concentrations of the extract as well as in the negative control (2% DMSO). In the figure **23(a)** depicted the negative control where only 2% DMSO solution was added. In the **Figure 23(b)**, it was seen that the cell viability decreased due to adding leaf extract of 0.0025mg/mL concentration; 7.89% cells growth inhibition was observed. The **Figure 23(c)** showed slightly less MOLT-4 cell viability in comparison to second picture as leaf extract of 0.025mg/mL concentration was added. In the fourth picture i.e. **Figure 23(d)** 0.25mg/mL concentration of

extract was added and a visible cell growth inhibition was observed which was approximately 30.76%. The **Figure 23(e)** showed cell viability at 2.5mg/mL plant extract concentration which showed highest cytotoxicity on MOLT-4 cell line. Lastly, **Figure 23(e)** depicted the 2.5mg/mL concentration of extract killing the maximum number of cells and exhibiting low cytotoxicity with almost 41.29% of cell growth inhibition as the IC_{50} value was found to be 3.29mg/mL.

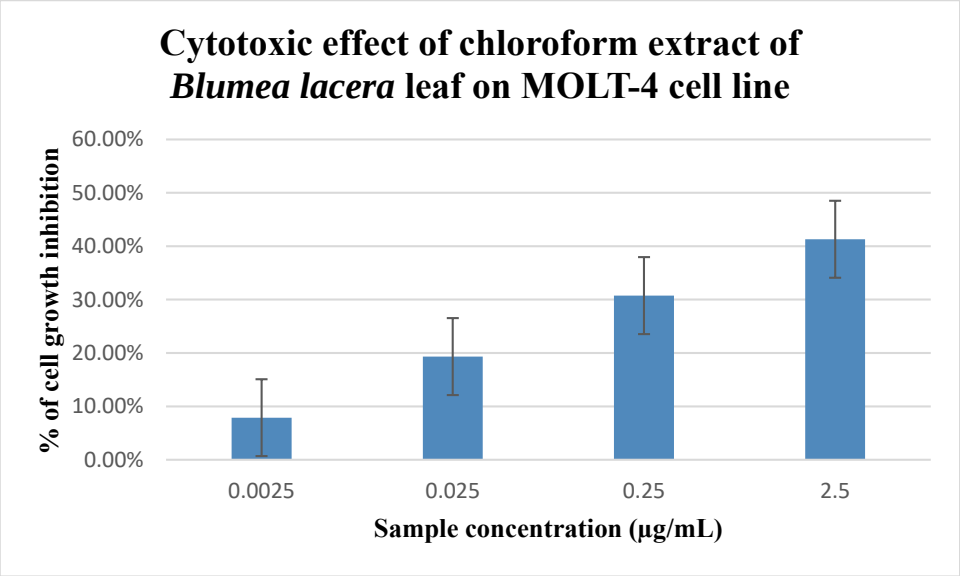


Figure 24: Cytotoxic effect of chloroform extract of Blumea lacera leaf on MOLT-4 cell line.

Interpretation: Figure 24 was the graphical representation of the percentage of cell growth inhibition versus the respective concentrations of plant extract. From the bar chart representation, it was very much evident that the lowest percentage of cell growth inhibition was observed at 0.0025mg/mL concentration of the plant extract depicting a greater cell viability. On the other hand, the lowest cell viability and the greatest percentage of cell growth inhibition was observed at 2.5mg/mL concentration of plant extract.

Chapter 5

Discussion

Blumea lacera is an Asteraceae family medicinal herb. This plant has a broad habitat, extending from Africa to Northern Australia, Guam and tropical Asia. Various parts of this plant has anti-inflammatory, diuretic, antioxidant, anthelmintic, antipyretic, antipyretic, antimicrobial (POI) activity, febrifuge, inhibitory anti-diabetic and α-amylase inhibitory activity (Rath et al., 2017; Salahuddin et al., 2015). Literature review showed that, specifically the leaves of *Blumea lacera* contain cytotoxic, anti-leukemic, antioxidant properties that helps to suppress the growth of cancer cells in our body (Akter et al., 2015).

Chloroform was used as solvent for the extraction process because of its polarity. Chloroform is a polar solvent with a dipole moment of 1.02. In fact, polar solvents had been used commonly for extracting polyphenols from plant extracts which was responsible for containing antioxidant and cytotoxic properties (Do et al., 2014). Previous investigations have showed that methanol extracts of *Blumea lacera* leaf of contained alkaloid, flavonoids, terpenoids and polyphenols (Akter et al., 2015). Chloroform us good for the extraction of high molecular weight flavonoids, alkaloids and polyphenols (Dai & Mumper, 2010). As a result, chloroform solvent was very suitable for antioxidant and cytotoxicity study of this plant.

Previous studies and experiments had found chloroform extract with alkaloid carbohydrate, flavonoids, glycoside, saponin, steroid, tannin, amino acid and terpenoids after several phytochemical screening of *Blumea lacera* leaves (Khandekar et al., 2013). The existence of these components provided us with an opportunity to understand and anticipate the various pharmacological functions. For example, antioxidant activity is exhibited by flavonoids and phenol, whereas cytotoxic activities are exhibited by terpenoid, saponins and tannins

(Aliomrani et al., 2017; Olatunji et al., 2019). Never before the chloroform extract of leaves of *Blumea lacera* used for estimating the antioxidant study, that made the necessity for the study of antioxidant potential in this project. In this study, the antioxidant potential of chloroform extract of *Blumea lacera* leaf had been observed *in-vitro* with different analytical techniques like-DPPH free radical scavenging assay, total flavonoid content (TFC), total phenolic content (TPC) and total antioxidant capacity (TAC). This study had provided quantitative data that confirms the presence of different phytochemicals, which led to antioxidant activities.

In the DPPH free radical scavenging assay, it had been found that highest percentage of free radical inhibition (about 86.43%) occurred in correspondence to the 1200µg/mL of concentration for *Blumea lacera* leaf chloroform extract. IC_{50} value for the standard (Ascorbic acid) and sample had been found 1.32µg/mL and 2.39µg/mL respectively. As a result, low concentration of the extract was required to inhibit the 50 % of DPPH free radicals although the value was slightly lower, that was comparable to the standard. It depicted that chloroform extract of *Blumea lacera* leaf possessed high antioxidant potential. Gallic acid, Quercetin and Ascorbic acid were used as standard in assay of total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) respectively. Different quantity of contents had been found for different concentration of extracts in those assays. For example, in case TPC, high phenolic contents were found in different concentration. To be specific, highest amount of phenolic content (285.47mg as GAE) per gram of plant extract had been found for 1200µg/mL of concentration. Again, in case of TFC, highest flavonoid content (285.47mg as QE) per gram of plant extract had been found for 1200µg/mL of concentration. Furthermore, highest antioxidant capacity (272.21mg as AAE) per gram of plant extract had been found for 1200µg/mL of concentration. Previously, no literature review had been found comprising the evidence of antioxidant studies for *Blumea lacera* leaf

chloroform extract. However, in case of methanol extract of *Blumea lacera* leaf, literature review showed that the extract had IC_{50} value of 19.9µg/mL, which was much higher than the value found in this study for chloroform extract (A. Rahman et al., 2013). Therefore, strong DPPH free radical scavenging activity, phenolic contents, flavonoid contents and antioxidant capacities can be attributed to the fact that the leaf extract possess strong antioxidant potential (Badarinath et al., 2010).

In-vitro cytotoxicity test known as the MTT assay on cervical cancer cell (HeLa) and T-cell acute lymphoblastic leukemia (MOLT-4) cell was performed to investigate the cytotoxic activities of *Blumea lacera* leaf for different concentrations of chloroform extract taking the cell viability into consideration. Percentage of cell growth inhibition was determined where 2% DMSO was used as the negative control for each cell line.

In a previous study, methanol extract of 16 Bangladeshi medicinal plants were investigated for their cytotoxicity study against three human cancer-cell lines (breast: MDA-MB-435S; gastric: AGS and colon: HT-29) using the MTT assay, where highest cytotoxicity (IC_{50} 0.01–0.08 mg/mL) was found for methanol extract of *Blumea lacera* leaf against all cell lines (Tiralongo et al., 2011). In another study, methanol extract of *Blumea lacera* leaf showed strong cytotoxicity and produced IC_{50} value of 2.62µg/mL against MCF-7 cells, which was human cancer cell line (Akter et al., 2015). Cytotoxicity for different species of *Blumea* such as- *Blumea balsamifera*, *Blumea lanceolaria*, *Blumea eriantha* and *Blumea mollis* had been studied before against different cell lines. All of these species showed high cytotoxicity (Lalmuanthanga et al., 2019). However, chloroform extract of *Blumea lacera* leaf had not been studied before for cytotoxic activity against any cell lines. Literature studies showed that chloroform extract of leaves of this plant possessed various kind of phyto-constituents and as a result it created the scope to explore the cytotoxic activities of this extract. The pictures showed that the cell death increased as the concentration of plant extract was increased. As

the concentration of the plant extract began increasing in the cell culture, number of cells begun to decline. Against the HeLa cell line, the highest percentage of cell growth inhibition (39.17%) occurred at 2.5µg/mL of concentration for chloroform extract of the plant leaf with IC_{50} value of 3.46 mg/mL. This showed low cytotoxic activity for the extract. The image of negative control was taken to identify the difference of cell growth inhibition and viable cells among five of the concentrations where survival of cells was 100% and no cell death was notified. The inhibition of cell growth of the cancer cells was concentration dependent. As the concentration of the extract increased, it showed higher amount of cell growth inhibition. At the highest concentration, chloroform plant extract showed highest cytotoxic activity, as a percentage of cell growth inhibition was only 31.44%. However, the plant extract showed low cytotoxicity against HeLa cell line. Against the MOLT-4 cell line, the highest percentage of cell growth inhibition (41.29%) occurred at 2.5µg/mL of concentration for chloroform extract of *Blumea lacera* with IC_{50} value of 3.29mg/mL. This showed low cytotoxic activity for the extract though the highest concentration of the extract showed more toxicity against MOLT-4 cell line in comparison to HeLa cell line. The image of negative control was also taken to identify the difference of cell growth inhibition and viable cells. It was observed that as the concentration of the extract increased, it showed higher amount of cell growth inhibition. There may be error in the result due to different physiological and environmental factors, also variation in concentration. By comparing the cytotoxicity of chloroform extract *Blumea lacera* leaf against HeLa and MOLT-4 cells, it was observed that the extract had slightly more cytotoxicity against MOLT-4 cell line.

Lastly, chloroform extract of *Blumea lacera* had shown strong antioxidant activity and low cytotoxic activity. As a result, the plant may be used to treat oxidative degenerative diseases, such as Alzheimer's disease and Parkinson's disease. Further study will be focused on compound isolation so that some novel compounds can be identified for therapeutic purpose.

Chapter 6

Conclusion

Among several *in-vitro* antioxidant assays used, DPPH free radical scavenging assay gave IC_{50} value of 2.39µg/mL for chloroform leaf extract compared to 1.32µg/mL for standard Ascorbic acid. All of the *in-vitro* antioxidant techniques used in this project namely- DPPH free radical scavenging assay, total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) exhibited strong antioxidant potential. As a result, overall antioxidant potential was strong. Cytotoxic potential assessed using MTT assay for the chloroform extract of *Blumea lacera* leaf against HeLa and MOLT-4 cell line denoted IC_{50} value of 3.46mg/mL against HeLa cells and 3.29mg/mL against MOLT-4 cells that indicated low cytotoxic potential. Thus, leaf extract this plant may be beneficial to treat oxidative various degenerative diseases.

Chapter 7

Future Prospects

The current research showed that *Blumea lacera* chloroform extract has good antioxidant activity relative to ascorbic acid and moderate cytotoxic activity. As a result, it extends to a whole range of distinct scopes for further research:

1. Cytotoxicity investigation using other cancer cell lines like lung cancer, prostate cancer, breast cancer *etc.* can be carried out as other cell lines were not used to study cytotoxic potentials earlier.
2. Bioactive compound isolation can also be performed for determination and quantification with high performance liquid chromatography (HPLC), mass spectroscopy, infrared spectroscopy and NMR (nuclear magnetic resonance) of certain structural bioactive compounds. The analysis of these compounds is also possible.
3. Information of *in-vitro* antioxidant activity of *Blumea lacera* leaf opens up another option for *in-vivo* research of antioxidants to show the therapeutic effect of experimental animal models.
4. Additional pharmacological research has not yet been undertaken on antimicrobial, anti-viral and anti-pyretic properties. These study proposals could lead to the discovery of drugs, the isolation of medicines and could serve as a natural source for the production of novel drug compounds.
5. Information of antioxidant capabilities can contribute to an understanding of the structure of the molecules engaged in further research. With the use of *Blumea lacera* chloroform extracts, synergistic results with other extracts can be correctly demonstrated.

Chapter 8

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