Investigation of *In-vitro* Antioxidant and Cytotoxic Potential of 1:1 Mixture of *Grape Seed* and *Green Tea* Ethanolic Extract

A project submitted

by

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Dedicated to my mother, Mrs. Khorsheda Khanam for her love and support...

Certification Statement

This is to certify that the project titled "Investigation of *In-vitro* Antioxidant and Cytotoxic Potential of 1:1 Mixture of *Grape Seed* and *Green Tea* Ethanolic Extract" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Raushanara Akter**,** Associate Professor of the Department of Pharmacy, BRAC University. Throughout the project, I have given appropriate credit where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the Supervisor,

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Acknowledgement

I would like to start by thanking the Almighty for enabling me to carry out this research in good health and frame-of-mind. Secondly, I would like to state that two persons have played pivotal role in my life; one of them is my mother who has shaped my thought process and the other is my project supervisor, Dr. Raushanara Akter, Associate Professor, Department of Pharmacy, BRAC University.

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Abstract

Grape seed extract and Green tea (also known as *Camellia sinensis*) are two very common plants extracts that have been generally used in several food and beverage applications. Due to the health benefits associated with them, makes them a popular choice for our study. From various literature reviews, it has been revealed that these plants exhibit properties such as anti-oxidant, hypoglycemic, anti-microbial, anti-cancer, anti-smoking, have food safety applications and also provide synergistic effects when consumed with certain drugs such as 5-Fluorouracil. Since no *in-vitro* antioxidant and cytotoxic potential studies were carried out with the 1:1 mixture of grape seed and green tea extract in ethanolic solvent, this study was designed to observe whether their anti-oxidant potential and cytotoxic activity increases due to synergistic effects or not. Firstly, phytochemical screening revealed that the *Grape seed extract (GSE)* did not exhibit alkaloid and glycosides. Similarly, *Green tea extract (GTE)* showed the absence of alkaloid, saponin and glycosides. For better understanding of the antioxidant potential of 1:1 mixture of *GSE* and *GTE*, several *in-vitro* antioxidant tests were carried out. The DPPH free radical scavenging assay denoted that 96.42% of inhibition of free radicals was evident at 1200µg/mL of the extract. The IC_{50} was found to be 75.86µg/mL for the plant extract, whereas the IC_{50} was found to be 84.783µg/mL for the standard ascorbic acid which indicated that the extract exhibited more anti-oxidant activity than the standard. The Total Phenolic Content (TPC) was found to be 122.87 mg of gallic acid equivalent (GAE)/g at 1200µg/mL. The Total Flavonoid Content (TFC) and the Total Antioxidant Capacity (TAC) was found to be very low. Therefore, overall anti-oxidant potential was moderate. Cytotoxic activity was examined using an *in-vitro* cytotoxicity screening method known as MTT assay on cervical cancer cell line (HeLa). The results indicated that highest percentage of cell growth inhibition was seen for 2.5mg/mL concentration of sample in which about 37.97% of cell growth was inhibited giving IC_{50} value of 8.1 mg/mL. To conclude, it can be inferred that due to absence of synergistic effect, the ethanolic extract of 1:1 mixture of grape seed and green tea showed weak cytotoxic effect and moderate antioxidant potential.

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List of Abbreviations

AA: Ascorbic Acid

AAE: Ascorbic Acid Equivalent

CAT: Catalase

DPPH: 1, 1-Diphenyl-2-Picryl Hydrazyl

FCR: Folin-Ciocalteu Reagent

FRS: Free Radical Scavengers/Scavenging

GA: Gallic Acid

GAE: Gallic Acid Equivalent

GSE: Graph Seed Extract

GSPE: Grape seed proanthocyanidins extract

GTE: Green Tea Extract

GSH: Glutathione Peroxidase

GSSG: Glutathione Disulfide

HPLC: High Performance Liquid Chromatography

HPV: Human Papilloma Virus

LAF: Laminar Air Flow

QE: Quercetin Equivalent

RA: Rosmarinic Acid

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SDV: Standard Deviation

SOD: Super Oxide Dismutase

TAC: Total Antioxidant Capacity

TFC: Total Flavonoid Content

TPC: Total Phenolic Content

CHAPTER ONE

INTRODUCTION

Chapter 1: Introduction

1.1. Phytotherapy and its relation to medicinal plants

According to WHO, “A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis.” When a plant is denoted as ‘medicinal’, then the plant is assumed to be useful as a therapeutic agent or drug or a medicinal preparation’s active ingredient (Sofowora, Ogunbodede, & Onayade, 2013).

Commonly phytotherapy means the treatment in which natural plant-derived medicines are used. Today, plants and plant-derived medicines are one of the key sources of modern pharmaceuticals used to cure many diseases. There are roughly certain chemical constituents existing in the plants that exert discrete pharmacological activity on human body. These are also referred as bioactive compounds; among them phenolic compounds, flavonoids and alkaloids are considered to be the most significant (Devendra, Srinivas, & Solmon, 2012).

1.2. Medicinal plants in drug discovery

The countless bioactive compounds in medicinal plants and their derivatives are solely responsible for employing diverse types of pharmacological actions leading to the discovery of new drugs. Two aspects serve as the base for screening and developing new drugs from plants; namely biodiversity and chemical structure diversity.

Medicinal plants are comprised of numerous bioactive compounds and contain large amount of structurally diverse natural products compared to synthetic compounds. Natural products express mostly advanced binding characteristics than synthetic compounds. That indicates that bioactive compounds have greater capability to interact with other molecules; noteworthy attribute for an effective drug (Lahlou, 2013). Some of the medicinal plants with their traditionally use and pharmacological activity are listed in **Table 1.1**.

Table 1.1: Some medicinal plants used for treating common diseases (Rahman et al., 2001).

Source	Used Plant Parts	Local Name	Pharmacological activity	Traditional use
Abroma augustac Linn (Sterculiaceae)	Leaf	Ulatkambal	Male antifertility	Amenorrhoea
Achyrathus asperae Linn. (Sterculiaceae	Aerial part	Apang	Antidiabetic	Diuresis
Eclipta albaa Linn. (Compositae)	Tuber	Keshraj	Antihypertensive	Stomachic
Momordica charanteaf Linn. (Cucurbitaceae)	Fruit	Corolla	Antidiabetic	Diabetes
Persicarie astagninag Linn. (Polygonaceae)	Whole plant	Biscatali	Analgesic	Pain

From the **Table 1.1**, it is very clearly evident that corresponding to a plant, the specific part that is used is mentioned followed by its local name, activity and traditional use. As for example, for the plant *Abroma augustac,* the leaves are used to treat amenorrhoea and its basic pharmacological activity is male infertility. It is very essential to state the plant part because different parts of the same plant exhibit different types of properties and one part of the plant may exhibit more biologically active potential than other parts

1.3. Natural products as remedies

Approximately 120 different chemical substances at present are derived from the diverse plants with medicinal properties and are consequently used as drugs around the world. Chemical substances like tannin, alkaloid, flavonoid etc. express medicinal and when present in certain amounts are believed to be used for the treatment of certain diseases. For example, phyto-constituents owning anti-oxidant properties are thought to prevent or slow down diseases such as cancer (J. Lee, Koo, & Min, 2004).

Natural bioactive compounds are highly diverse in accordance to the role in plant metabolism. Among them alkaloids, glycosides, flavonoids, phytosterols, phenolic compounds, phenols, saponin, steroids and tannins are considered to be directly related to countless healing properties (Mamta Saxena, Saxena, Nema, Singh, & Gupta, 2013).

Table 1.2 Some of the important bioactive compounds with their actions and natural sources (J. Lee et al., 2004).

Drug/chemical constituent	**Pharmacological action**	**Plant source**
Aescin	Anti-inflammatory	*Aesculushippo castanum*
Bromelain	Anti-inflammatory, proteolytic	*Ananas comosus*
Convallatoxin	Cardiotonic	*Convallaria majalis*
Demecolcine	Antitumor agent	*Colchicum autumnale*
Digitalin	Cardiotonic	*Digitalis purpurea*
Emetine	Amoebicide, emetic	*Cephaelisipecacuanha*
a-Lobeline	Smoking deterrant, respiratory stimulant	*Lobelia inflate*

Table 1.2 highlights some of the drugs that are isolated from natural sources with their basic pharmacological action. For example, Digitalin obtained from *Digitalis pupurea* exhibits cardiotonic properties and therefore can be used for cardiovascular diseases.

1.4. Antioxidants

Both natural and synthetic antioxidants are used to treat many diseases and to avert cellular damages caused by reactive oxygen species or free radicals. The medicinal plants exhibiting antioxidant properties generally contain polyphenols and flavonoid, a polyphenolic chemical.

1.4.1. Classification of antioxidants

Antioxidants can be classified primary, secondary and tertiary antioxidants according to their function. Primary antioxidants stop the oxidant formation. The secondary antioxidants scavenge ROS/RNS while tertiary antioxidants cause restoration of the oxidized molecules (Halliwell, 2012).

Antioxidants can be classified into six categories grounded on their mode of actions (Flora, 2009) which are discussed in the **Table 1.3**.

Table 1.3: Classification of antioxidants based on mode of actions stated in (Flora, 2009).

Mode of action	**Examples of antioxidants**
Antioxidant that break chains by reacting with peroxyl radicals having weak O-H or N-H bonds.	Phenol, Naphthol
Antioxidants that break chains by reacting with alkyl radicals	Quinones, Nitrones, Iminoquinones
Antioxidants that decompose hydroperoxide	Sulphide, Phosphide, Thiophosphate
Antioxidants that deactivate metals	Diamine, Hydroxyl acids, Bifunctional compounds
Antioxidants that terminates the cyclic chain	Aromatic amines, Nitroxyl radical, Variable valence metal compounds
Antioxidants that act synergistically with other antioxidants	Phenol sulphide in which the phenolic group reacts with peroxyl radical and sulfide with hydroperoxide

The **table 1.3** shows that naphthols and phenols function by terminating free radical reaction via reaction with peroxyl radicals. On the contrary, nitrones, iminoquinones, and quinones stops the free radical reaction via reacting with alkyl radicals. Other methods include deactivating metals, decomposing hydroperoxide, and accomplishing synergistic effect through other antioxidant use and finally terminating the cyclic chain.

1.4.2. Enzymatic and non-enzymatic antioxidants

“Antioxidants are largely classified to enzymatic and non-enzymatic antioxidants. The antioxidant enzymatic system directly or indirectly contributes to defense against the ROS.

Figure 1.1 provides the broad classification of enzymatic and non-enzymatic antioxidants. explain the figure content

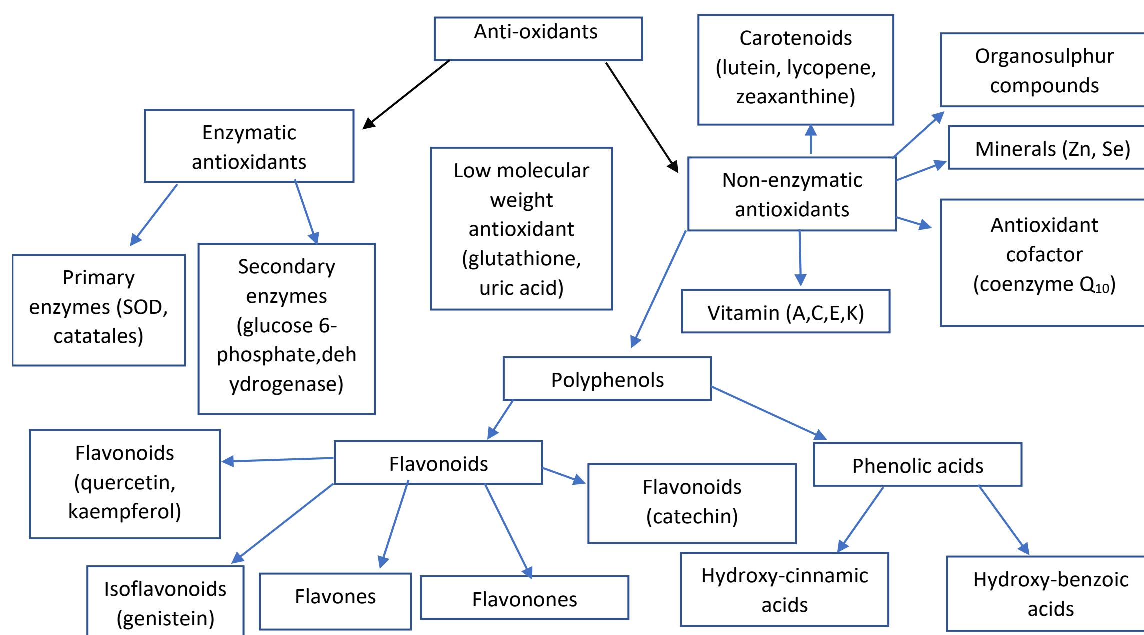


Figure 1.1: Enzymatic and non-enzymatic antioxidants (Mehta & Gowder, 2015).

The **Figure 1.1** explains that enzymatic antioxidants are classified into primary and secondary which are further classified into primary, secondary enzymes under enzymatic antioxidants. Non-enzymatic antioxidants are also classified into carotenoids, vitamins, minerals, organosulphur and polyphenols.

Table 1.4: Major enzymatic and non-enzymatic antioxidants along with their location and properties (Mehta & Gowder, 2015).

Enzymatic antioxidants	**Location**	**Properties**
Superoxide dismutase (SOD)	Mitochondria and cytosol	Dismutation of superoxide radicals
Catalase (CAT)	Mitochondria and cytosol	Remove hydrogen peroxide
Glutathione Peroxidase (GSH)	Mitochondria and cytosol	Remove hydrogen peroxide and organic hyperoxide

Non-enzymatic antioxidants	**Location**	**Properties**
Vitamin C	Aqueous phase of cell	Acts as a free radical scavenger and recycles vitamin E
Vitamin E	Cell membrane	Major chain breaking antioxidant in cell membrane
Uric acid	Product of purine metabolism	Scavenger of OH radical

The **Table 1.4** shows the particular locations and properties of antioxidants. Enzymatic antioxidants are found in cytosol and mitochondria of the cells. Especially enzymatic antioxidants are present mostly inside the cells. Non-enzymatic antioxidants have diversified locations and also their properties also have huge variations like vitamin E found in the cell membrane and favors in chain breakage reactions.

1.4.3. Phytochemicals exerting antioxidant activity

Natural antioxidant compounds instead of synthetic antioxidant compounds must be investigated and used if possible. Vitamin C, vitamin E possess antioxidant potential but phenolic acids and flavonoids exhibit higher antioxidant. Polyphenols, present in plants are characterized by one or more hydroxyl moieties and aromatic ring(s). Polyphenols are categorized into four main classes including flavonoids, lignan, stilbenes, and phenolic acids (M. T. Lee, Lin, Yu, & Lee, 2017). The reaction between free radicals and such compounds causes (**Figure 1.2**), the gain of electron which gets delocalized over phenolic antioxidant. This inhibits the free radical chain reaction by stabilizing the aromatic nucleus's resonance effect. The mechanism of such a reaction is shown on the **Figure 1.2**.

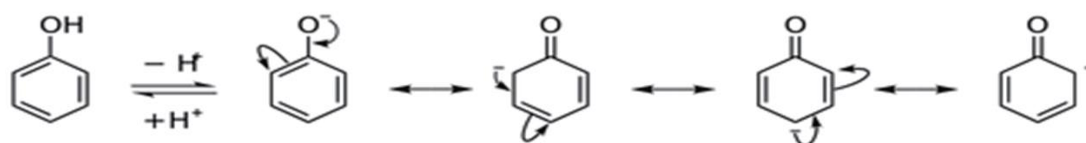


Figure 1.2: Resonance of aromatic nucleus of antioxidants (M. T. Lee et al., 2017).

1.5. Free radical and reactive oxygen species

Free radicals are produced as a result of normal cellular aerobic metabolism in the biological systems. In some cases, unusual chemical reactions can generate them that are stimulated by certain xenobiotics and diseases. Whereas reactive oxygen species (ROS) is used to denote reactive species together – Singlet oxygen (O_2), Alkoxyl radical ($RO^{\cdot}$), Superoxide anion radical ($O_2^{\cdot-}$), Peroxyl radical ($ROO^{\cdot}$), Hydrogen peroxide (H_2O_2), Hydroxyl radical (OH) and Lipid hydroperoxide (LOOH). Reactive nitrogen species (RNS) are in addition accountable for some free radical reactions; $^{\cdot}NO$ and $ONOO^{-}$,respectively. Many radicals are very reactive and can extract an electron or donate an electron to other molecules. Therefore, acting as oxidants or reductants (Young & Woodside, 2001).

1.5.1. Mechanism of tissue damage by free radicals

The free radical chain reaction mechanism depends on their interaction with protein, lipid or DNA base. The interaction of free radicals or ROS/RNS with protein, lipid and DNA, it results in chemical reactions and numerous changes inside the cells; leading to cell and tissue injury. **Figure 1.3**, the pathway to cell and tissue injury by free radical reaction is explained (James P. Kehrer & Klotz, 2015).

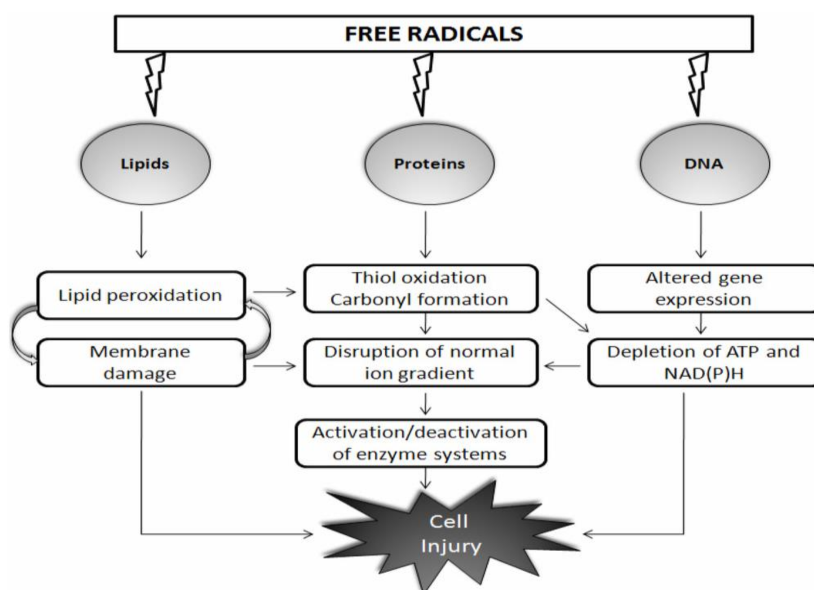


Figure 1.3: Mechanism of cell injury by free radicals (James P. Kehrer & Klotz, 2015).

In the **Figure 1.3**, cell and tissue injury via free radical reaction is elaborated. If free radicals react with lipid molecules, lipid peroxidation occurs which causes membrane damage and injury. Again, protein molecules reaction with ROS/RNS's results in enzymes activation that harms the cell. Finally, free radicals react with DNA which damages DNA through alteration and mutation of genetic expression (James P. Kehrer & Klotz, 2015).

1.5.2. Antioxidant defense system against free radicals

Radicals have the capability to react in a way that can lead to any cellular component damage. Because of this, both exogenous and endogenous types of antioxidant defense systems function to protect cellular components from induced free radical damage. The defense system species participating can be classified into three major groups: chain breaking antioxidants, transition metal binding proteins and antioxidant enzymes.

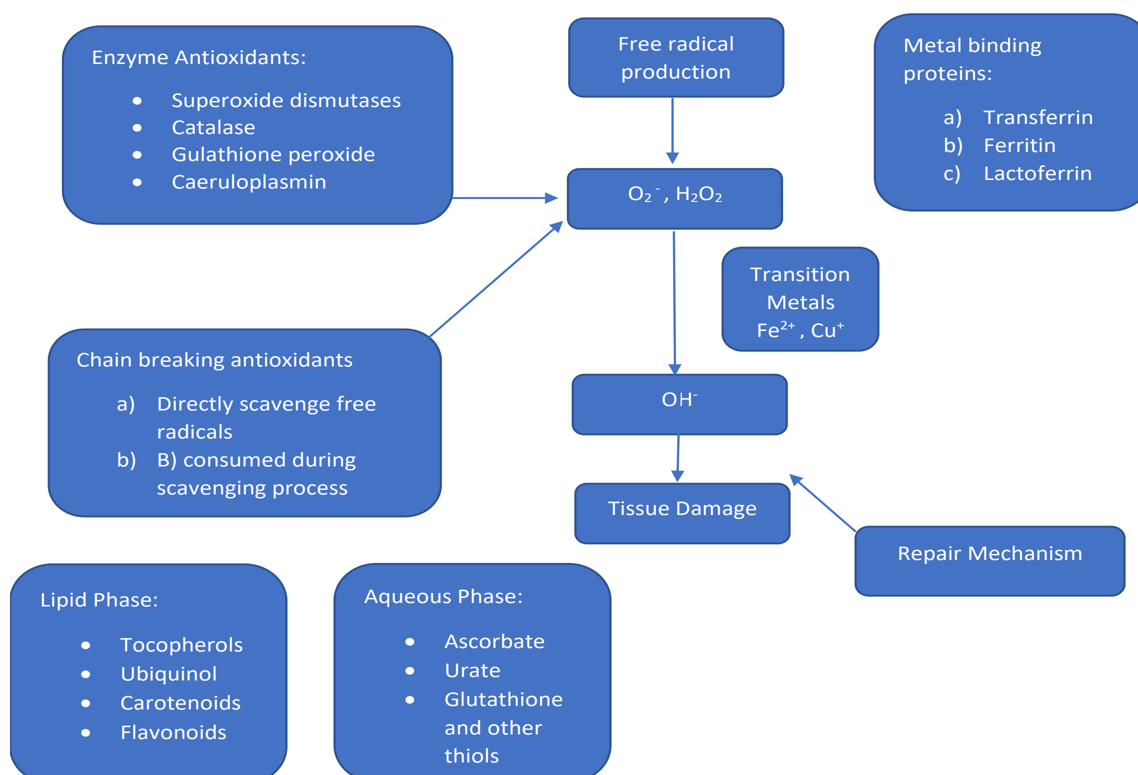


Figure 1.4: Antioxidant defense against free radical attack (J P Kehrer, 1993).

In the **Figure 1.4** antioxidant defense mechanism is elucidated. Antioxidant enzymes catalyze free radical species breakdown. Transition metal binding proteins stop the communication of transition metals such as copper or iron with superoxide and hydrogen

peroxide producing highly reactive hydroxyl radicals. Chain breaking antioxidants being strong electron donors, react with free radicals causing damage to target molecules. Thus, as the antioxidant is being oxidized, it should be replaced or regenerated.

1.6. Phytochemicals

Medicinal plants exhibit various diverse chemical constituents, the presence and quantity can differ from plant to plant. Additionally, different plant parts may carry different grades and extent of phytochemicals. It is vital to identify the phytochemicals to comprehend its pharmacological actions (M. T. Lee et al., 2017). The major groups of phytochemicals include alkaloid, phytosterol, tannin, flavonoid, phenolic compound, saponin, carbohydrate and glycoside which are discussed in **Table 1.5**.

Table 1.5: Major Phytochemicals and their biological functions (M. T. Lee et al., 2017).

Class of Phytochemicals	Mechanism of action	Example
Alkaloid	Alkaloids act as topoisomerase (I) inhibitor and interacts with tubulin. Some of them target the mitochondria of a cancer cell, they show cytotoxicity effect in different stages of cell proliferation.	Arecoline (*Areca catechu*) Atropine (*Atropa belladonna*) Digitoxin (*Digitalis purpurea*)
Flavonoids	They can inhibit or minimize the cellular damage caused by free radical reactions. These compounds possess antimicrobial, estrogenic, anti-allergic and, cytotoxic antitumor activity.	Flavones Flavonols Flavanones
Phenol and Phenolic compounds	They contribute to their inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways.	Stilbenes Lignins Phenolic acids

Carbohydrate	Carbohydrates serve as injectable anticoagulant, trigger the creation of antibodies, provide hormones etc.	Heparin Amylopectin
Phytosterol	Phytosterols have been found to inhibit proliferation, induce apoptosis, and reduce invasiveness of cancer cells in culture.	Campesterol Stigmasterol
Tannin	Tannins are capable of forming reversible and irreversible complexes with proteins, alkaloids, nucleic acids and minerals.	Gallotannin Ellagitannin
Glycoside	These compounds are potent inhibitors of cellular Na^+/K^+-ATPase.	*Digitalis purpurea*

1.7. *In-vitro* antioxidant activity study methods

Both in food and *in-vitro*, oxidation reactions are complex. This is one of the reasons as to why more than one antioxidant tests needs to be conducted to measure the antioxidant capacity. In addition, the mechanism of action in the laboratory test tubes and inside the human body might vary due to presence of absence of certain factors. As a result, few *in-vitro* antioxidant testing assays are used to calculate antioxidant capacity (Badarinath et al., 2010).

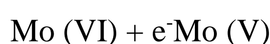
1.7.1. DPPH free radical scavenging assay

To determine the "free radical scavenging activity" of the antioxidant in a sample plant extract, DPPH (1, 1-diphenyl-2-picryl-hydrazyl) is used. DPPH produces stable free radicals in methanol or any aqueous solution via the delocalization of the free electrons. This in turn generates a deep purple color solution which has characteristic absorption at 517nm. DPPH gets reduced to hydrazine in presence of antioxidants/hydrogen donors. This reaction changes the color of the solution from purple to yellow which is characteristic change. As a result, reduction in absorbance of DPPH at 517nm is directly related to or is proportional to the scavenging activity. This is indicative by noticing the intensity of color change from purple to yellow that can be visually observed (Raquibul Hasan et al., 2009).

1.7.2. Determination of total phenolic content (TPC)

Polyphenols act as hydrogen or electron donors and as a result their antioxidant property depends on their ability to stabilize and/or delocalize unpaired electron which can break the chain in order to inhibit the free radical reaction. Also, they can form chelate metal ions that stops the Fenton reaction (Rice-Evans, Miller, & Paganga, 1997).

This method measures the reduction capacity using folin-ciocalteu reagent (FCR). The compound in FCR is assumed to be a heteropolyphosphotunstates-molybdate that goes through a series of reduction reactions where transfer of one or two electrons forms a blue-colored complex, possibly $(PmoW_{11}O_{40})^{-4}$. The reaction that takes place is as follows:



$$Mo\ (VI) + e^{-} Mo\ (V)$$

The absorbance is measured at 765nm against blank (ethanol and reagents) using the UV-Visible Spectrophotometer. Then total phenolic content (C) is measured in terms of gallic acid equivalents (GAE).

1.7.3. Determination of total flavonoid content (TFC)

Flavonoids can scavenge free radicals, inhibit enzymes that bring about free radical reaction and cause chelation of metal ions like copper and iron. Based on the chemical structure of flavonoids, various mechanisms can be employed for scavenging free radicals (Benavente-Garcıa, Castillo, Marin, Ortuño, & Del Río, 1997).

This method intends to determine the total flavonoid content by a stable acidic complex that is formed between 3 substrates – the C-4 keto-group with either the C-3 or C-5 hydroxyl group of the flavones and flavanols present in the sample with that of aluminium chloride (Pal, Sannigrahi, & Mazumder, 2009). Furthermore, aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of the flavonoids. The intensity complex formation is measured spectrophotometrically at 415nm.

1.7.4. Determination of total antioxidant capacity (TAC)

Also known as the phosphomolybdenum method, the total antioxidant capacity can be determined quantitatively using this method following spectrophotometric analysis at

695nm. The determination of anti-oxidant potential is based on the reduction of Mo (VI) to Mo (V) by analyte. As a result, green-colored phosphate-molybdenum (V) complex is formed at an acidic pH.

1.8. Cervical cancer

Cervical cancer is one of the major causes of cancer deaths in women. About 260,000 women die of cervical cancer and among them 85% deaths occur in deveoping countries. Whereas WHO estimates that among 500,000 new cases of cervical cancer anually, 80% women are affected by it whose age is between 15 and 45 years residing in developing countries. HPV (Human Papilloma Virus) is responsible for the cervical cancer occured in cervix, as well as cancer of vagina, vulva, penis and anus, along with some head and neck cancers, angeogenital warts and recurrent respiratory papillomatosis (Nour, 2009).

1.8.1. Human papilloma virus and its' mechanism to cause cervical cancer

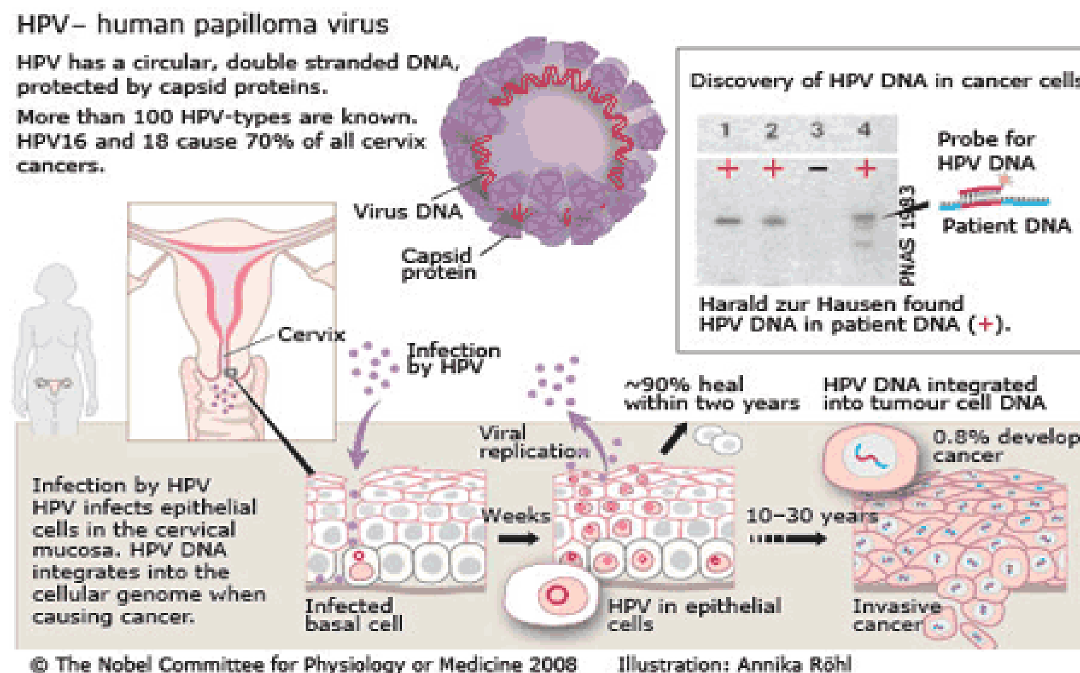


Figure 1.5: The mechanism of HPV attacking human cell and causing cervical cancer (Nour, 2009).

The **Figure 1.5** shows the HPV attack on human cell. After entering the body, HPV effects the cervical mucosa's intraepithelial layer. Almost half the patient's intake antibiotics and

seroconversion take place in about 8-12 months. Seroconveriosn is period when antibody develops and becomes detectable in blood stream. the infected cell forms adenocarcinoma in-situ (AIS) or cervical intraepithelial neoplasia (CIN). Squamous cell carcinoma or adenocarcinoma can result if untreated.

1.8.2. Risk factors for cervical cancer

The virus causing cervical cancer is a sexually transmitted virus. The major risk factors are listed below (Nour, 2009):

(a) Early age at first sexual activity

(b) Smoking

(c) Immunosuppression (HIV)

(d) Long term use of oral contraceptives

(e) Multiple sex partner

(f) Early age at first delivery

(g) Increased number of pregnencies

1.8.3. Treatment and prevention of cervical cancer

Among women cervical cancer is the third most common malignancy. The selection of drugs and treatment strategies relies on the stage of cancer. Thus, it can be termed as "stage-specific". To treat the metastatic cancer, reagents such as topotecan, paclitaxel, carboplatin, ifosfamide and cisplatin. Their chemical structures are given in **Figure 1.6** (Ordikhani et al., 2016).

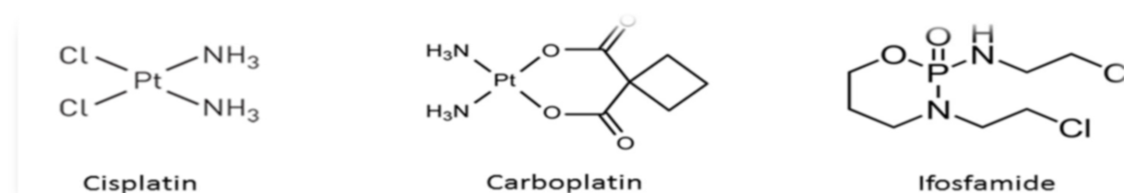


Figure 1.6: Chemical structure of anticancer drugs used to treat cervical cancer (Ordikhani et al., 2016).

Cisplatin is the most effective chemotherapeutic agent for advanced cervical cancer treatment. it functions by interacting with DNA and forming adducts. This stops gene transcription that causes appoptosis in tumor cells and oxidative stress. Preventive HPV

vaccines against cervical cancer are also made avilable (Lowy, Solomon, Hildesheim, Schiller, & Schiffman, 2008).

1.9. Cytotoxic activity

Cytotoxicity refers to the property of a chemical substance that can cause cell death. The application of a cytotoxic agent to a cancerous or normal cell will cause cell necrosis. If an agent kills both normal and cancerous cells, then it is a cytotoxic agent. On the other hand, if an agent kills only cancerous cells and not normal cells, then it can be a potential anticancer agent. Therefore, it is essential to identify cell viability and cytotoxic assay of an agent to assess its ability to harm cancerous cells and normal cells.

1.9.1. MTT (3 - (4,5 – dimethylthiazol-2-yl) - 2, 5 - diphenyltetrazolium bromide) assay

The assay of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is a kind of tetrazolium reduction assay which is a broadly used assay to find cell viability. The first step is to prepare the MTT substrate in a solution. Then it is added to cultured cells and finally it was incubated for a period of 1 to 4 hours. The theory shows the quantity of produced formazan is proportional to the number of viable cells. The measurement is taken by measuring the changes in absorbance at 570 nm. This is carried out using a plate reading spectrophotometer (Riss et al., 2013).

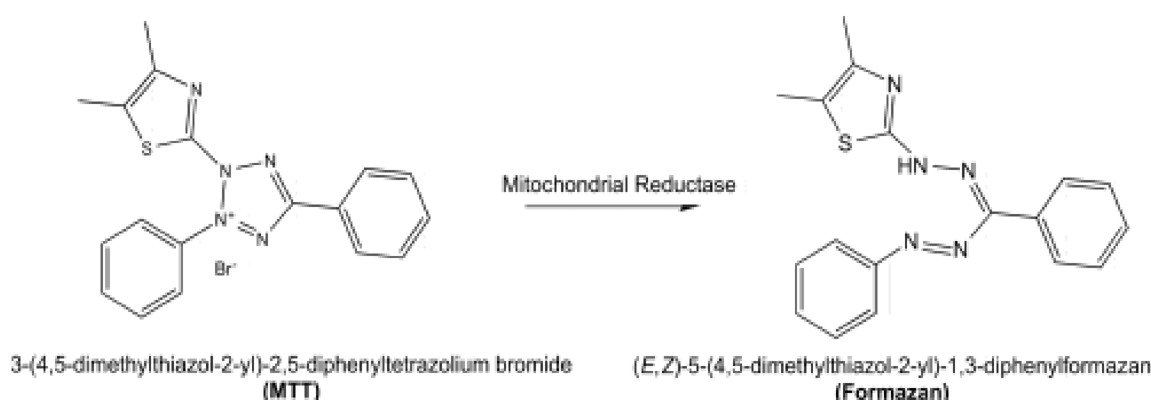


Figure 1.7: Structure of MTT and colored formazan product (Riss et al., 2013).

1.10. Overview of Bangladeshi medicinal plant

It is estimated that almost 87% of drugs are sourced from nature and nearly 25% of the drugs that are prescribed are made from plants. Around 80% people for their primary health care in developing countries are rely on herbal or plant-based medicines. These plants show many pharmacological activities such as, antioxidant, antibacterial, anti-inflammatory antifungal, anticancer, etc. Bangladesh holds a rich source of medicinal plants of different variety. The diverse ecology and traditional resources are two key reasons for this. In order to formulate traditional medicines, approximately 250 species of medicinal plants are used in many parts of Bangladesh (Khatun et al., 2014). Some medicinal plants with their traditional uses are discussed in the **Table 1.11** below.

Table 1.11: Some medicinal plants with traditional (Khatun et al., 2014).

Plant name	**Local name**	**Traditional use**
Adhatoda vasica	Basak	Cough
Cinnamomum zeylanicum	Dalchini	Vomiting
Eugenia jambolana	Jam	Dysentery
Helitropium indicum	Hatissur	Pyresis
Psidium guajava	Piyara	Diarrhoea

In the **Table 1.11,** the plant name corresponding to its local name and traditional use is provided. For example, Piyara is actually *Psidium guajava* used commonly for diarrhea and Hatissur, scientifically known as *Helitropium indicum* is used to treat pyresis.

1.10.1. Selection of *Grape Seed Extract (GSE) and Green Tea Extract (GTE)* for the present study

After doing extensive literature review on medicinal plants of Bangladesh, it was found that a Grape seeds and Green tea extracts were previously studied for cytotoxicity using different cell lines. No study for the cytotoxic activity was found to be carried out using HeLa cell which is human cervical carcinoma cell line. Moreover, the study of antioxidant activity of a combination of grape seed and green tea have been conducted several times in many different solvents. Such as ethanolic extract and the 1:1 ratio of *GSE* and *GTE* have not been studies for their antioxidant study and therefore is a rare combination. That is why, the study of antioxidant activities with ethanolic extract of *GSE* and *GSE* along with cytotoxicity activity on HeLa cell line was planned for the present study.

1.11. Rationale of the project

GTE and *GSE* are two prevalent plant extracts that have been generally used in several food and beverage applications. Green tea has health benefits like antioxidant, antimicrobial, anticarcinogenic and anti-inflammatory properties. Grape seed also has antioxidant, antimicrobial and anti-inflammatory properties. These two plant extracts have antimicrobial activities against major food borne pathogens like *Campylobacter jejuni*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium* in preventing pathogenic contamination. In addition, they show probable antioxidant properties by preventing the lipid oxidation and have demonstrated synergism in antimicrobial activity when used in combination with organic acids such as benzoic acids, tartaric acid and malic acid. Since no *in-vitro* antioxidant and cytotoxic potential studies were carried out with the 1:1 mixture of grape seed and green tea extract in ethanolic solvent, thus this study was designed to observe whether their anti-oxidant potential and cytotoxic activity increases due to synergism. This study will also help to serve as a good base for finding out their particular properties and also aid in comparative study with their extracts in other solvents and ratios.

1.12. Aim of the project

Investigation of *in-vitro* antioxidant and cytotoxic potential of 1:1 mixture of *GSE* and *GTE*.

1.13. Objectives of the project

After studying the literature review pertaining to the previous findings of *GSE and GTE*, the objectives of the project were made as follows with regards to using 1:1 mixture of *GSE and GTE*:

- Carrying out phytochemical screening in order to qualitatively determine the presence of chemical constituents.
- Determination of its antioxidant potential using various *in vitro* methods and assessment of its free radical scavenging potential comparable to that of standard ascorbic acid using DPPH free radical scavenging method.
- Scope to compare antioxidant activity
- Investigation of cytotoxicity of ethanolic extract on HeLa cell line.

1.14. Introduction of the Plants

Green Tea:

Habitat: Green Tea (*Camellia sinensis*) was first originated in China several thousand years ago. They belong to a group of large shrubs or to a group of small trees.

Specifications: They contain leaves up to 10 cm in length, serrate, elliptic and coriaceous. They consist of flowers of diameter 3.75 cm, white peduncles along with few distinct bracts, at times a second flower in the axil of one of them and with depressed capsules of globose, 3-cornered, 3-seeded. The leaves have a length of 4–15 cm (1.6–5.9 in) long and breadth of 2–5 cm (0.79–1.97 in).

Constituents: Fresh leaves consist of about 4% caffeine. There are two important kinds of tea, green tea and black tea, and both contain caffeine (1–5%) with little amounts of other xanthine alkaloids. Tea composition varies with season, climate, age of the leaf, and tea variety. They also contain large quantities of phenolic substances or tannins (5–27%). Phenolic substances include gallic acid units and catechin (flavanol) with those in green

tea being higher in amounts than those in black tea. Generally, green tea leaves contain 36% polyphenols, with pronounced amounts of catechin. Pharmacological activities of tea are due mainly to its alkaloids (caffeine) and catechins, which are classified into four main compounds: epicatechin gallate (ECG), epicatechin (EC), epigallocatechin gallate (EGCG), epigallocatechin (EGC), and four secondary compounds, catechin (C), catechin gallate (CG), gallocatechin (GC), and gallocatechin gallate (GCG). EGCG is the major catechin in green tea leaves (48–55% of total polyphenols) (Wanasundara & Shahidi, 1998). Other constituents present in tea include amino acids, fats (4– 16%), vitamins, minerals, sterols, flavour and aroma chemicals, triterpenoids proteins, and others. Several reports have reported green tea as chemo-preventive and therapeutic properties (McKay & Blumberg, 2002). It has also been shown that tea possesses anticarcinogenic, antimutagenic, and anticlastogenic effects (Gupta, Saha, & Giri, 2002). For reasons mentioned above, green tea has become an alluring material for research.

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Magnoliopsida – Dicotyledons
Subclass Dilleniidae
Order Theales
Family Theaceae – Tea family
Genus Camellia L. – camellia P
Specie Camellia sinensis (L.) Kuntze –tea

Figure 1.8: Photograph of green tea leaves

Grape seed:

Grape seed extract, a nutritious food that is prepared from refined natural grape seeds. Grape seed extract is considered an efficient natural antioxidant. It cannot be synthesized within a person directly from the grape seed. The extract is stated as one of the strongest antioxidants. The antioxidant property is 20 times that of vitamin C and 50 times of that of vitamin E.

Classification and properties: It effectively remove additional free radicals within the human body and has an important role in slowing the aging process and it also enhances immunity function. Anti-oxidant, anti-fatigue, strengthening, anti-hypersensitivity, improving sub-health, slowing the aging process, improving irritability, memory loss and dizziness. Most grapes are obtained from the cultivars of Vitis vinifera, which is a the European grapevine native to Central Asia and the Mediterranean. Minor amounts of wine and fruit come from Asian and American species such as:

- *Vitis amurensis*, the most important Asian species.
- *Vitis labrusca*, the North American table and grape juice grapevines (including the Concordcultivar), sometimes used for wine, are native to the Eastern United States and Canada.
- *Vitis mustangensis*, (the mustang grape) found in Mississippi, Alabama, Louisiana, Texas, and Oklahoma.
- *Vitis riparia*, a wild vine of North America, is sometimes used for winemaking and for jam. It is native to the entire Eastern U.S. and north to Quebec.
- *Vitis rotundifolia* (the muscadines) used for jams and wine, are native to the Southeastern United States from Delaware to the Gulf of Mexico.

Kingdom	**Plantae – Plants**
Subkingdom	**Tracheobionta – Vascular plants**
Superdivision	**Spermatophyta – Seed plants**
Division	**Magnoliophyta – Flowering plants**

Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae
Order	Rhamnales
Family	Vitaceae – Grape family
Genus	*Vitis* L. – grape
Species	*Vitis amurensis* Rupr. – Amur grape

Traditional use: Grape is considered one of the largest fruit crops, which has an annual production of about 58 million metric tons. Phenolics, a component in red wines and grapes have been reported to lower human low-density lipoprotein (LDL) oxidation in vitro (Teissedre, Frankel, Waterhouse, Peleg, & German, 1996). Grape seeds are rich in monomeric phenolic compounds, such as, (−)-epicatechin, (+)-catechins and (−)-epicatechin-3-*O*-gallate, and also dimeric, trimeric besides tetrameric procyanidins, and these compounds play an important role as antiviral agents and antimutagenic (Saito, Hosoyama, Ariga, Kataoka, & Yamaji, 1998). It is also suggestive that possible use of phenolics in grapes aid in inhibiting atherosclerosis.

Chemical constituents: The major compounds include (+)-catechin (11%), (−)-epicatechin (10%), epicatechin 3-*O*-gallate-(4β→8)-catechin (B1-3-*O*-gallate) (7%), epicatechin-(4β→8)-epicatechin (dimer B2) (6%), and (−)-Epicatechin-3-*O*-gallate (9%). It was reported monomers of (−)-epicatechin, (+)-catechin, and (−)-epicatechin-3-*O*-gallate, one tetrameric procyanidin, 11 trimeric procyanidins and 14 dimeric from grape seeds. Significant quantities of extremely polymerised procyanidins are present in grape seeds. It has been reported that about 55% of the procyanidins in grape seeds have more than 5 monomer units (Fuleki & Ricardo-Da-Silva, 2003).

Figure 1.9: Photograph of grape seed

CHAPTER TWO:
LITERATURE REVIEW

Chapter 02: Literature Review

2.1. Anti-oxidant properties:

The Grape seed proanthocyanidins extract (GSPE) reduced H_2O_2-induced oxidant stress in cardiomyocytes. Antioxidant action is linked with a rise in cardiomyocyte survival and contractile function. The supplementation of a meal with GSPE minimizes the postprandial oxidative stress by decreasing the oxidants and increasing the antioxidant levels in plasma. Similarly, green tea polyphenol is known to be an excellent antioxidant that directly scavenges free radicals and inhibits lipid peroxide formation. Green tea extract has been shown to protect against cardiovascular and renal diseases in several *in vitro* and *in vivo* models (Stangl, Lorenz, & Stangl, 2006). Green tea catechins delay the oxidation reactions by inhibiting the formation of free radicals or interrupting the propagation of the free radical chain reaction caused by the toxic compounds (Ding et al., 1992).

In another study, total phenolics and antioxidant activities of Grape seed and green tea were analyzed. HPLC (high performance liquid chromatography) was used for their phenolic constituent determination and it ranged from 24.8 to 92.5 mg of chlorogenic acid equivalent/g dry material determined by the Folin-Ciocalteu method. The antioxidant activities of methanolic extracts investigated by conjugated diene measurement of methyl linoleate were 3.4-86.3% and seven phenolics in grape seed and green tea extracts were identified that ranged from 15.38 to 1158.49 and 18.3 to 1087.02 mg of chlorogenic acid equivalents /100 g of extract, respectively (Rababah, Hettiarachchy, & Horax, 2004). To prevent or reduce lipid oxidation in food products, green tea and grape seed extracts can be use.

Various antioxidant behaviors using CAT assay and ApoCAT assays in complex food systems were carried out in a study. The relationship between hydrophobicity and antioxidant potential was a positive relationship as per the results. Green tea extract mixed with tocopherols showed a higher antioxidant capacity in the ApoCAT assay than in the CAT assay, while rosemary extracts and grape seed extracts did not exhibit considerably different changes in behaviors in both assays (Panya et al., 2015).

The effect of metal chelators, natural hydrophilic antioxidants and hydrophilic antioxidant/metal chelator mixture on the oxidative stability of water-in-algae oil emulsion and base algae oil was examined in a study. The results exposed that ascorbic acid and green tea extracts had utmost protective effect against algae oil oxidation (B. Chen, Rao, Ding, McClements, & Decker, 2016). On the other hand, grape seed extract, did not exhibit any noteworthy protective effect. Natural antioxidants and ascorbic acid did not exhibit any synergistic effect.

2.2 Effects on and when combined with other drugs:

Midazolam: The effects of 15 herbal extracts in dietary supplements on CYP2C9, CYP3A4 and CYP2D6 activities in human liver microsomes was investigated. CYP activities were strongly inhibited by in vitro addition of green tea extracts (*GTE*) or grape seed extracts (*GSE*). To observe the effects of these extracts on CYP3A activities in vivo, the pharmacokinetics of midazolam (MDZ) was analyzed in rats. These studies show that *GTE* or *GSE* possibly will alter the pharmacokinetics of MDZ after subchronic ingestion. GTE effect on CYP3A activity seem to be differing between liver and small intestine (Nishikawa et al., 2004).

Leucovorin/ 5-fluorouracil: MB-6 is a botanical preparation composed of fermented green tea extract, soyabean extract, grape seed extract, Antrodia camphorata mycelia, curcumin and spirulina extract. MB-6 combined with leucovorin/5-fluorouracil was used for chemotherapy in a rodent study. Thus, 72 patients with metastatic colorectal cancer were randomly grouped and made to receive the anticancer drug with MB-6 and another group received the drug therapy without MB-6 i.e placebo group. After 16 weeks of study and up to 77 weeks of follow up study, the results indicated that patients in the MB-6 group had a considerably lesser disease development rate than patients in the placebo group (0.0% vs 15.8%, P = .026). The placebo group had a pointedly greater incidence of adverse events compared to the MB-6 group (28.9% vs 2.9%, respectively, P = .004). In conclusion, it can be said that MB-6 is a botanical supplement that may increase the usefulness of chemotherapy in patients with metastatic colorectal cancer (W. T. L. Chen et al., 2014).

Methotrexate: The cytogenotoxicity of methotrexate which is an anti-neoplastic drug itself is alarming. In order to minimize the risk associated with methotrexate, some micronucleus assay was conducted on various bioactive phytochemicals and their combinations. The results indicated that of all the bioactive phytochemical combinations, the combination of grape seed proanthocyanidins and eleutherosides from Siberian ginseng as well as green tea polyphenols and eleutherosides exhibited stronger antimutagenic effects; the inhibition rate of methotrexate-induced genotoxicity separately reached $74.7 \pm 6.5\%$ and $71.8 \pm 4.7\%$ (Gu, Wu, & Yang, 2016). Therefore, it could be concluded that combinations of bioactive phytochemicals have the probability to be used as cytoprotectors.

2.3 Hypoglycemic activity:

One study evaluated the inhibitory effects of green tea, white tea, grape seed and catechins on α-amylase and α-glucosidase. These are two glucosidases that are needed for starch digestion in humans. To assess the relative potency of plant extracts and catechins, their concentrations required for 50 and 90% inhibition of enzyme activity were determined. Then it was compared with acarbose, pharmacological glucosidase inhibitor. Results showed that grape seed extract strongly inhibited both α-amylase and α-glucosidase activity whereas catechin 3-gallates and tea extracts were poor inhibitors of α-amylase but strong inhibitors of α-glucosidase (Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012).

2.4 Anti-microbial activity:

Grape seed and green tea show antimicrobial activities against pathogens such as Campylobacter jejuni, Listeria monocytogenes, Escherichia coli O157:H7, and Salmonella Typhimurium. Pathogenic bacteria contaminate poultry which leads to human foodborne disease. Irradiation is a safe means of decontaminating poultry products but might interfere with sensory quality characteristics. A combination of tartaric acid (TA), grape seed (GS) and green tea (GT) extracts were vacuum-infused into chicken breast fillets, followed by irradiation at 1, 2, and 3 kGy by electron beam irradiation. A test group was also incorporated to investigate the impression, texture, flavor, appearance, and tenderness. The results depicted that samples vacuum-infused with TA at 37.5 and 75.0 mM and irradiated at 1 kGy significantly reduced Listeria monocytogenes (L.m.) whereas vacuum-infusion of TA at 37.5 and 75.0 mM at 2 and 3 kGy irradiation, reduced L.m. to near nondetectable

levels (Over et al., 2010). The addition of TA, GS and GT to chicken breast fillets lead to the prevention of L.m. contamination but did not significantly impact consumer tenderness, appearance, preference or flavor.

2.5 Anti-cancer activity:

Growth-related and cancer-specific cell surface NADH oxidase alongside protein disulfide-thiol interchange activity designated tNOX from human cervical carcinoma (HeLa) cells, transplanted tumors in mice and mouse mammary 4T1 cells in culture were used for anticancer investigation of grape extracts in combined and alone forms. Grape skins extract in ethanol were more effective source than either juice, pulp or seeds. The grape extracts mixed with decaffeinated green tea extracts synergistically inhibited tNOX activity and cancer cell growth. Green tea extract mixed with ground freeze-dried pomace in the ratio of 25:1 when injected intratumorally was effective in inhibiting growth of 4T1 mammary tumors in situ in mice (Morré & Morré, 2006).

The effect of the mixture of Grape seed and Green tea on radiation induced immune suppression in rats. The ratio of *GTE* to *GSE* was 1:2 and the investigation was conducted on about 5 groups of rats with one of them being control. The results depicted that the groups treated with the *GSE* and *GTE* mixture depicted an increase in concentrations of immune cells such as CD4 and CD8. The level of pro inflammatory cytokines Tumor Necrosis Factor-α and C-Reactive Protein raised up after γ-irradiation and expressively lessened by mixture administration (El-Desouky, Hanafi, & Abbas, 2017). In addition, groups subjected to antioxidant mixture showed increase in all haematological parameters and a significant decrease in cholesterol and triglyceride levels. Therefore, it could be concluded that *GTE* and *GSE* mixture is a decent immune modulator compound and radioprotector, signifying its likely use as an adjuvant during radiotherapy.

2.6 Food safety and applications in the food industry:

Green tea extract *(GTE)* and grape seed extract *(GSE)* are two popular plant extracts that have been commonly used in numerous food and beverage applications. Green tea and grape seed exhibit anticarcinogenic, antioxidant, antimicrobial and anti-inflammatory properties. In addition, they show antimicrobial activities against pathogens such as *Campylobacter jejuni, Listeria monocytogenes, Escherichia coli O157:H7,* and

Salmonella Typhimurium (Perumalla & Hettiarachchy, 2011). They play great role in food safety and quality. Therefore, nanoscale delivery of active compound from such plant materials using electrostatic sprays can enhance the food quality and safety which is a green solution.

Green tea *(GTE)* and grape seed *(GSE)* extracts are anticipated as preservatives for increasing the shelf life of low sulphite raw beef patties. The antimicrobial and antioxidant activities of *GST* and *GSE* were compared with ascorbate. Ascorbate, *GST* and *GSE* enhanced the preservative effects of SO_2 on beef patties (Bañón, Díaz, Rodríguez, Garrido, & Price, 2007). This study proposed that the amount of SO_2 supplementary can be reduced to attain better raw meat products.

The quality of red drum (*Sciaenops ocellatus*) fillets were examined using grape seed extract (*GSE)* and tea polyphenols (TP), combined with chitosan (Ch), as natural preservatives during refrigerated storage. Control was prepared with two different treatments (Ch+GS and Ch+TP). The sampling was done at 0, 4, 8, 12, 16, 20 days and stored at (4 ± 1°C) for 20 days. Physicochemical, microbiological and sensory attributes were sporadically evaluated. It was reported that shelf life was extended by 6-8 days compared with the control (Li, Li, Hu, & Li, 2013). In addition, quality was effectively and properly maintained during storage.

2.7 Anti-smoking potentiality:

A probable cigarette filter composed of glutathione (preferably L-glutathione) and green tea and/or grape seed extract is capable of neutralizing and scavenging the free radicals originating from heated tobacco and passing through the filter as the smoker inhales. This is further capable of reducing free radical damage to the respiratory tract, oropharyngeal cavity and lungs (Hersh, Us, Hersh, Us, & Wittenberg, 2002).

CHAPTER THREE

METHODOLOGY

Chapter 3: Methodology

3.1. Collection and authentication of plant material

After numerous studies about the two plants and having reviewed many literatures, it was strongly seen that there have been a lot of studies conducted on these two plants individually and in combination as well. Fortunately, ethanolic solvent in the 1:1 ratio of mixture was not used. Therefore, that lead to the construction of the research as such. The grape seeds were collected from fresh grapes by scraping off the flesh. On the other hand, green tea was obtained from commercially available packets.

3.2. Extraction procedure

The entire extraction procedure can be virtually divided into 2 parts:

(a) Plant material preparation and drying (2-steps)

(b) Extraction process (5-steps)

3.2.1. Plant material preparation and drying

The steps involved in the extraction of ingredients from the crude medicinal plant. First the grape seeds were plucked off from grapes and washed in distilled water. The seeds were then left to dry in a sir tight bag and not under the sunlight to prevent photo degradation.

3.2.2. Extraction process

3.2.2.1. Size reduction and weighing

The dried, crispy grape seeds and green tea leaves were then separately grounded to coarse powder using a high capacity grinding machine. This was followed by packaging into air-tight plastic containers with necessary labeling which were finally left in a cool, dry and dark place until further investigation. During the grinding process, necessary measures were taken to avoid cross-contamination. The powdered grape seed and green tea were taken in two individual beakers and then the total weight was weighed in a weight machine.

3.2.2.2. Extraction

For the purpose of this study, the maceration process of extraction was used for extraction of plant materials and ethanol was utilized as the organic solvent. The two beakers containing powdered grape seed and green tea material was soaked in 900mL of ethanol separately for a period of 7 days at normal room temperature (22-25°C) with occasional agitation. The outcome of maceration process was a 2-layer phase: the lower-most phase is the sediment and the upper-most is a cloudy suspension of plant extracts in ethanol.

3.2.2.3. Filtration

After seven days of maceration, the contents of the beaker were separately decanted first before filtering them using Whatman filter (pore size: 110mm).

3.2.2.4. Concentration and Drying

The collected filtrate was concentrated using rotary evaporator (Heidolph) at 100rpm at 30°C, until concentrated ethanolic extract is produced. Thereafter, the mixture was transferred into a beaker for drying under Laminar Air Flow (LAF). Finally, the beaker was placed under LAF to evaporate the solvent from the extract, leaving behind dry and semi-solid extract. LAF was used as a preventive measure so as to avoid any chances of microbial growth on the extract while it's drying. After successful drying of the extract, the beaker was covered in Aluminum foil and refrigerated for further use.

3.3. Phytochemical screening

Phytochemical screening was performed on the crude extracts of grape seed and green tea separately in order to access its qualitative chemical compositions namely, alkaloids, carbohydrates, tannin, flavonoids, glycosides, etc.

3.3.1. Detection of alkaloid

For the qualitative determination of alkaloids, three tests were performed. 0.5g of ethanolic extract of grape seed and 0.5g of ethanolic extract of green tea were separately dissolved in 5mL of 1% Hydrochloric acid, boiled in a water bath followed by filtration. Using the filtrate obtained the following tests were performed individually on the *GST* and *GSE*:

3.3.1.1. Hager's test

To 2mL of the filtrate, a few drops of Hager's reagent (1% picric acid solution) was added and the presence of alkaloids were confirmed by the formation of yellow precipitate (Waldi, 1965).

3.3.1.2. Mayer's test

A 10mL Mayer's Reagent is prepared by dissolving 0.1358g of Mercuric (II) Chloride and 0.5g of Potassium Iodide in 10mL distilled water. Then, to a 2mL of the filtrate, a few drops of Mayer's reagent were added along the sides of the test tube. The formation of a white or creamy precipitate indicates the presence of alkaloids (Rice-Evans et al., 1997).

3.3.1.3. Wagner's test

A 10mL Wagner's Reagent is prepared by dissolving 0.2g of Iodine crystals and 0.6g of Potassium Iodide in 10mL distilled water. To a 2 mL of the filtrate, a few drops of Wagner's reagent were added. Formation of a brownish-black precipitate confirms the presence of alkaloids in the sample (Steinke, Müller, & Wagner, 1993).

3.3.2. Detection of carbohydrate

Carbohydrate can by qualitatively detected by weighing out 0.5 g of ethanolic extract of 1:1 mixture of *GST* and *GSE* and dissolving it in 5 mL of distilled water and then filtering the mixture (Ramakrishnan, Prasannan, & Rajan, 2001). To the filtrate obtained, the following two tests were performed.

3.3.2.1. Molisch's test

2mL of the filtrate obtained was treated with 2 drops of Molisch's Reagent i.e. alcoholic solution of α-napthol to which 2mL of concentrated sulfuric acid was pipetted along the sides of the test tube and was allowed to stand for a while. The formation of a violet ring indicates the presence of carbohydrates (Ramakrishnan et al., 2001).

3.3.2.2. Fehling's test

To 2 mL of the filtrate, 1mL of each of the Fehling's solution A and B were added in a 1:1 ratio and then boiled for a few minutes. Formation of a brick-red precipitate indicates the presence of reducing sugar (Ramakrishnan et al., 2001).

3.3.3. Detection of flavonoid

3.3.3.1. Lead acetate test

The ethanolic extract was treated with a few drops of lead acetate solution and the formation of yellow colored precipitate signifies the presence of flavonoids (Sindhu S, Uma, Manorama, & Sindhu, 2013).

3.3.3.2. Zinc ribbon test

The presence of flavonoids can be confirmed by another method. To a test tube containing 0.5mL of alcoholic extract, 5-10 drops of concentrated Hydrochloric acid and a small piece of Zinc was added. The solution was then boiled for a few minutes and then left to stand. The formation of a red to crimson color solution indicates the presence of flavonoids (Sindhu S et al., 2013).

3.3.4. Detection of phenols/phenolic content

3.3.4.1. Ferric chloride test

This test is performed by measuring 2mL of extract in a test tube followed by adding 3-4drops of 15% (w/v) Ferric chloride solution. The formation of a bluish-black precipitate signifies the presence of phenols (Soni & Sosa, 2013).

3.3.5. Detection of phytosterols

3.3.5.1. Libermann Burchard's test

To a small amount of extract, 1 L of chloroform was added and filtered. The filtrate was then treated with a 2mL of acetic anhydride, boiled and cooled. Finally, 1mL of concentrated sulfuric acid was added to the solution. Formation of a brown ring at the junction indicates the presence of phytosterols (Soni & Sosa, 2013).

3.3.6. Detection of steroids

3.3.6.1. Salkowski test

To 1mL of extract, 2mL of chloroform, 1mL of sulfuric acid were added. The appearance of red color indicates the presence of steroids (Ghani, 1998).

3.3.7. Detection of tannins

3.3.7.1. Lead acetate test

To 1mL of the extract, a few drops of 1% Lead acetate solution were added and the formation of a yellow-colored precipitate indicates the presence of tannins (Prashant Tiwari, Kumar, & Gurpreet Kaur, 2011).

3.3.7.2. Potassium dichromate test

10% Potassium Dichromate solution is prepared by dissolving 1g of Potassium Dichromate was dissolved in 10mL distilled water to prepare this solution. 5mL aqueous solution of crude extract was dissolved in 1mL of 5% ferric chloride solution and the formation of yellow precipitation indicates the presence of tannin (Ghani, 1998).

3.3.7.3. Ferric chloride test

5% Ferric chloride solution is prepared by dissolving 0.5g of ferric chloride in 10mL distilled water.5mL aqueous solution of crude extract was dissolved in 1mL of 5% ferric chloride solution and the formation of greenish black precipitation indicates the presence of tannin (Ghani, 1998).

3.3.8. Detection of resins

Presence of resin can be identified by adding 5-10 drops of acetic anhydride to 2mL of the extract and heating the solution gently. This is then followed by addition of 0.5mL of sulfuric acid to the solution. Presence of resin is identified by the formation of a bright purple color (Soni & Sosa, 2013).

3.3.9. Detection of glycosides

The ethanolic extract of green tea and grape seed separately were hydrolyzed with dilute Hydrochloric acid before subjecting it to Borntrager's Test (Senthilkumar, 2013).

3.3.9.1. Borntrager's test (modified)

To the 5 L of filtrate, 5mL of 5% Ferric (III) chloride and 5mL of dilute Hydrochloric acid were added. This was followed by heating the mixture for 5 min in a boiling water-bath and cooling it down. Then, 5mL of benzene was added to the mixture and shaken thoroughly. The organic layer was then separated by using a separating funnel and an equivalent volume of dilute ammonia solution was added. The formation of a pinkish-red color in the ammonical layer signifies the presence of glycosides (Kamalakar, Prabhakar, & Shailaja, 2014).

3.3.10. Detection of saponin

3.3.10.1. Froth test

The extract is diluted with distilled water and the volume was made up to 20mL and the contents of the cylinder were shaken in a graduated cylinder for about 15min. The formation of a foam layer of about 2cm in height indicates the presence of saponins (Kamalakar et al., 2014).

3.4. *In-vitro* antioxidant activities

There are various *in vitro* methods of determining anti-oxidant activities of a plant extracts. Of the various methods, four methods were chosen to determine the antioxidant activity in plant extract of *1:1* mixture of *GSE* and *GTE*, namely DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging assay, total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC).

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

The DPPH free radical scavenging assay of ethanolic extract of *GST* and *GSE* mixture was determined using the method described by (Raquibul Hasan et al., 2009).

3.4.1.1. Reagents and chemicals

The reagents and chemicals that are required to carry out the determination of DPPH scavenging activity is listed below in **Table 3.1**.

Table 3.1: Reagents and chemicals used to determine DPPH scavenging activity

Name of reagent/chemical	**Source**
DPPH	Sigma Aldrich, U.S.A.
Ethanol	Active Fine Chemicals Ltd., Bangladesh
L-ascorbic acid	Merck, Germany

3.4.1.2. Reagent preparation

0.004% (w/v) DPPH solution was prepared by dissolving 2mg of DPPH in 50mL distilled water and then stored in the refrigerator at -4°C till before use.

3.4.1.3. Sample and standard solution preparation

120mg (60mg *GSE* and 60mg *GTE*) of extract was measured and dissolved in 10mL of ethanol to produce a concentration of 12mg/mL. This became the sample stock solution.

The sample concentrations were prepared by serial dilution of the sample stock solution to derive 8 serially diluted concentrations: 1200, 800, 400, 200, 100, 50, 25 and 12.5µg/mL.

The standard used was L-ascorbic acid and it was prepared in the same manner as the extract resulting in eight serially diluted concentrations, ranging from 1200-12.5 µg/mL.

3.4.1.4. Experimental procedure

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

(b) To each of the test tube, 2mL of 0.004% (w/v) DPPH solution was added.

(c) Then, the test tubes were incubated for 30 minutes at room temperature which was followed by measuring the absorbance of the resulting solutions and control (DPPH and methanol) at 517nm against blank (Ethanol) using a spectrophotometer (U-2910 UV-Vis Spectrophotometer).

(d) The percentage of free radical scavenging activity (% FRS) was then calculated from the equation as follows:

$$\text{\% Free radical Scavenging (\%FRS) activity} = \frac{(A0 - A1)}{A0} \times 100$$

Where, A_0 = The absorbance of the control

A_1 = The absorbance of the sample/standard

(e) Finally, the % Scavenging activity was plotted against concentration from which IC_{50} value was calculated (IC_{50} value is defined as the concentration at which 50% of total DPPH free radical is reduced by the antioxidants which can be determined by plotting % inhibition against the corresponding concentrations).

3.4.2. Determination of total phenolic content (TPC)

The TPC of the plant extract of mixture of *GST* and *GSE* was determined by the modified Folin-Ciocalteu method (Wolfe, Wu, & Liu, 2003).

3.4.2.1. Reagents and chemicals required

The reagents and chemicals that are required to carry out the determination of total phenolic content is listed below in **Table 3.2**.

Table 3.2: Reagents and chemicals used to determine total phenolic content

Name of reagent/chemical	**Source**
Folin-Ciocalteu reagent (FCR)	LOBA Chemicals Pvt. Ltd., India
Gallic acid monohydrate (Standard)	Sigma Aldrich, USA
Sodium carbonate	Merck Specialties Pvt. Ltd., Mumbai
Ethanol	Active Fine Chemicals Ltd., Bangladesh

3.4.2.2. Reagent preparation

20mL of 10% Folin-Ciocalteu reagent (FCR) solution was prepared by taking 2mL of FCR in a volumetric flask and diluting it with distilled water.

100mL of 7.5% (w/v) Sodium carbonate was prepared by measuring out 7.5g of sodium carbonate in a 100mL volumetric flask and then diluting it with distilled water to 100mL mark.

3.4.2.3. Sample and standard preparation

120mg (60mg *GSE* and 60mg *GTE*) of extract was measured and dissolved in 10mL of ethanol to produce a concentration of 12mg/mL. This became the sample stock solution. The sample concentrations were prepared by serial dilution of the sample stock solution to derive four serially diluted concentrations: 1200, 800, 400 and 200μg/mL.

Gallic acid was used as the standard and the stock solution was prepared in the same manner as the extract resulting in four serially diluted concentrations, ranging from 1200, 800, 400 and 200μg/mL.

3.4.2.4. Preparation of the blank

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

3.4.2.5. Experimental procedure

(a) 1mL of each of the fraction of sample and standard (gallic acid) concentrations was taken in test tubes.
(b) To which 2.5mL of FCR solution was added.
(c) 2.5mL of sodium carbonate solution was added.
(d) Each of the mixture was vortexed for 15s and then allowed to stand for 30min at 40°C in a water bath.
(e) Finally, the absorbance of standard and sample solutions was measured against blank at 765nm using spectrophotometer (U-2910 UV-Vis Spectrophotometer).

(f) The total phenolic content, C, for each of the fractions were expressed as Gallic Acid Equivalents (GAE) using the following equation:

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

Where, C = Total content of phenolic compounds, milligram of gallic acid per gram of dried plant extract, expressed as Gallic Acid Equivalent (GAE)

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

V = Volume of sample solution (mL)

m = Weight of the sample (g)

3.4.3. Determination of total flavonoid content (TFC)

The total flavonoid content of the extracts of *GSE and GTE* were determined according to the following method (Kumaran & Joel Karunakaran, 2007).

3.4.3.1. Reagents and chemicals required

The reagents and chemicals that are required to carry out the determination of total flavonoid content are listed below in **Table 3.3**.

Table 3.3: Reagents and chemicals used to determine total flavonoid content

Name of reagent/chemical	Source
Potassium acetate	Merck KGA, Germany
Quercetin (Standard)	Sigma Aldrich, Germany
Aluminum chloride	Merck Specialties Pvt. Ltd., Mumbai
Ethanol	Active Fine Chemicals Ltd., Bangladesh

3.4.3.2. Reagent preparation

100mL of 10% aluminum chloride solution was prepared by measuring 10g of aluminum chloride in a 100mL volumetric flask and diluting it with distilled water to 100mL mark.

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

3.4.3.3. Sample and standard preparation

120mg (60mg *GSE* and 60mg *GTE*) of extract was measured and dissolved in 10mL of ethanol to produce a concentration of 12mg/mL. This became the sample stock solution. The sample concentrations were prepared by serial dilution of the sample stock solution to derive 4 serially diluted concentrations: 1200, 800, 400 and 200µg/mL.

Quercetin was used as the standard and the stock solution was prepared in the same manner as the extract resulting in four serially diluted concentrations, ranging from 1200, 800, 400 and 200µg/mL.

3.4.3.4. Preparation of the blank

The blank solution contained 200µL of 10% aluminium chloride solution, 200µL of 1M potassium acetate solution, 5.6mL of distilled water and 4mL of ethanol, such that the final volume of the solution was 10mL.

3.4.3.5. Experimental procedure

(a) 1mL of each of the fraction of sample and standard (Quercetin) concentrations was taken in test tubes.
(b) To which 3mL of ethanol was added.
(c) And 200µL of 10% aluminium chloride solution and 200µL of 1M potassium acetate solution were added to each of the test tubes using 1000µL micropipette.
(d) Finally, 5.6mL of distilled water was added to the test to make the final volume of the solution 10mL.
(e) The test tubes were then incubated at room temperature for 30min.
(f) Afterwards, the absorbance of each of the sample and standard solutions were measured at 415nm against blank using spectrophotometer (U-2910 UV-Vis Spectrophotometer).
(g) The total flavonoid content, C, for each of the fractions were expressed as Quercetin equivalents (QE) using the following equation:

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

Where, C = Total content of flavonoid compounds, milligram of quercetin per gram of dried plant extract, expressed as Quercetin Equivalents (QE)

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

V = Volume of sample solution (mL)

m = Weight of the sample (g)

3.4.4. Determination of total antioxidant capacity (TAC)

The total Antioxidant capacity of plant extract of *GSE and GTE* were determined by using the following method (Prieto, Pineda, & Aguilar, 1999).

3.4.4.1. Reagents and chemicals required

The reagents and chemicals that are required to carry out the determination of total antioxidant capacity are listed below in **Table 3.4**.

Table 3.4: Reagents and chemicals used to determine total antioxidant capacity

Name of reagent/chemical	Source
Ammonium Molybdate	Active Fine Chemicals Ltd., Bangladesh
L-ascorbic acid (Standard)	Merck, Germany
Tri Sodium phosphate ($Na_3PO_4.12H_2O$)	Merck, Germany
Ethanol	Active Fine Chemicals Ltd., Bangladesh
Concentrated sulfuric acid (98%)	Merck, Germany

3.4.4.2. Reagent preparation

100mL of 0.6M Sulfuric acid was prepared by measuring 3.28mL of 98% concentrated sulfuric acid in a 100mL volumetric flask and diluting it with distilled water to 100mL mark.

100mL of 0.004M ammonium molybdate solution was prepared by measuring 4.494g of ammonium molybdate in a 100mL volumetric flask and diluting it with distilled water up to 100mL mark.

100mL of 0.028M trisodium phosphate solution was prepared by measuring 1.0645g of trisodium phosphate in a 100mL volumetric flask and diluting it with distilled water to 100mL mark.

3.4.4.3. Sample and standard preparation

120mg (60mg *GSE* and 60mg *GTE*) of extract was measured and dissolved in 10mL of ethanol to produce a concentration of 12mg/mL. This became the sample stock solution. The sample concentrations were prepared by serial dilution of the sample stock solution to derive 4 serially diluted concentrations: 1200, 800, 400 and 200µg/mL.

Ascorbic acid was used as the standard and the stock solution was prepared in the same manner as the extract resulting in four serially diluted concentrations, ranging from 1200, 800, 400 and 200µg/mL.

3.4.4.4. Preparation of the blank

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

3.4.4.5. Experimental procedure

(a) 300µL of each of the fraction of sample and standard (L-ascorbic acid) concentrations were taken in test tubes.

(b) 3mL of Reagent solution (0.6M sulfuric acid, 0.028M sodium phosphate and 0.004M ammonium molybdate) was added into the test tubes.

(c) The test tubes (sample, standard and blank) were then incubated at 95°C in a water bath for 90min.

(d) Finally, the absorbance of the sample and standard solutions was measured against blank at 695nm using spectrophotometer (U-2910 UV-Vis Spectrophotometer).

(e) The total antioxidant capacity, A, for each of the fractions were expressed as Ascorbic Acid Equivalents (AAE) using the equation as follows:

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

Where, C = Total antioxidant capacity, milligram of ascorbic acid per gram of dried plant extract, expressed as ascorbic acid equivalent (AAE)

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

V = Volume of sample solution (mL)

m = Weight of the sample (g)

3.5. Cytotoxic activity study by MTT assay

3.5.1. Solutions preparation

2% DMSO solution

2% DMSO solution was prepared by adding 60 µl in 2940 mL of distilled water for negative control.

3.5.2. 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.

3.5.3. Used Instruments

Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA), CO_2 Incubator (Nuaire, USA), Trinocular Microscope with Camera (Olympus, Japan), Hemocytometer.

3.5.4. Celltiter 96 assay kit

Celltiter 96 assay kit is an accumulation of qualified reagents that give a fast furthermore advantageous system with determination of the amount of proliferation and cytotoxicity (Ifere et al., 2010). It is a view of change of a cell division with tetrazolium salt under a formazan product that is undoubtedly distinguished utilizing a 96-well plate. A mixer of dye solution was added in 96 well plates to culture cells to perform this assay.

3.5.5. HeLa cell line

HeLa cell line was collected from Centre for Advance Research and Science (CARS). This cell line was cultured and maintained in DMEM (Dulbecco's Modified Eagles Medium) by using cell culture flask. It was stored in liquid nitrogen. DMEM is a widely accepted medium and used in many cell lines. It is high in glucose, vitamins and amino acids to help providing the necessary nutrition to cells.

3.5.6. Preparation of the different concentrations of plant extract

The assay was being performed by using five concentration 2.5 mg/mL, 2.0 mg/mL, 1.5 mg/mL, 1.0 mg/mL and 0.5 mg/mL of the 1:1 mixture *GST* and *GSE*. 20 mg/mL concentration was made by adding 20 mg of the extract (10mg *GSE* and 10mg *GTE*) in 1mL DMSO solution and it was the stock solution. 2.5 mg/mL was prepared by dilution the stock solution and then it was further diluted to prepare 2.0 mg/mL. 1.5 mg/mL concentration was made by diluting 2.0mg/mL solution by DMSO. In this way1.0 mg/mL and 0.5 mg/mL concentrations were made by serial dilution with. Then the samples were filtered through 0.45µm syringe filter prior to examination.

3.5.7. Cell Culture

3.5.7.1. Preparation of assay plates

HeLa cells were maintained in DMEM (Dulbecco's Modified Eagles Medium) in addition with 1% penicillin-streptomycin, 0.2% Gentamycin, 10% fetal bovine serum.

3.5.7.2. Thawing of cells

HeLa cells were preserved in liquid nitrogen in cryovials. The cryovial was taken and rapidly defrosted by swirling the vial delicately using a water bath at 37°C until there was a small piece of ice left in the vial. Afterward the thawed cells were transferred into a centrifuge tube which contained the DMEM medium drop wise under a laminar airflow hood. Thereafter, cells were suspended in the medium gently and transferred to the culture vessels.

3.5.7.3. Cell Passage

To get a fresh cell suspension cell passaging was done by transferring the cells into a new medium. The used cultured media was washed by FBS and followed by addition of 800μl of trypsin for detaching the cells from the top of the culture vessels. Then the cells were incubated and checked for the detachment under a microscope. After watching 90% of cells detached, 5 mL DMEM media was added to the vessels and blended using a pipette. Finally, 1 mL of this solution was taken and mixed with 4 mL of DMEM in a new vessel and kept in an incubator for further use.

3.5.7.4. Harvesting of cells

The cell was harvested using trypsin in log phase growth. Then cell was counted and seeded into 96 well plates.

3.5.7.5. Counting of cells

Cell counting had been carried out by using a hemocytometer. The hemocytometer was prepared by cleaning and polishing the mirror like surface deliberately with ethanol and lens paper. For adding the cell suspension, the coverslip was put in the counting surface. A pasteur pipet was used to introduce the fresh cell suspension into the hemocytometer. Enough suspension was presented so that the surface had been simply overflowed. Afterward the counting chamber was set in the microscope stage then the counting grid was focused. In a standard hemocytometer with neubauer rulings 1 entire grid can be observed at 40X magnification. The cells of the 4 large squares were counted. Either upper and left sides touching cells or lower or right sides touching cells were counted.

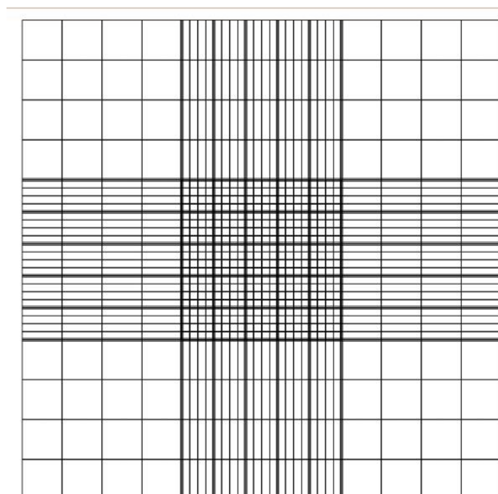


Figure 3.1: Hemocytometer

3.5.8. Procedure

Cytotoxic effect was performed in the Centre for Advanced Research in Sciences (CARS) using their services. The MTT colourimetric assay was performed by using celltiter 96 non-radioactive cell proliferation assay kit (Promega, USA). Cells were seeded onto 96 well plates and incubated at 37 °C and 5% of CO_2 atmosphere. After 24 hours of incubation, 10μL of sample was added into each well. Then it was again incubated for 2 days. After 2 days of incubation, cytotoxicity was examined using celltiter 96 non-radioactive cell proliferation assay kit. Then the absorbance was measured at 570nm using a 96-well plate reader. Same procedure had been followed for all 5 concentrations and negative control. Negative control was contained medium with 2% DMSO solution and blank was contained only medium. Duplicate wells were used for each sample. Cytotoxic activity was calculated by using a formula which is given below-

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

CHAPTER FOUR

RESULTS

Chapter 04: Results

4.1. Determination of percentage yield of the grape seed extract (*GSE*)

4.1.1. The total weight of powdered plant material

The table shows the initial weight of the powder along with the beaker and the total weight of the powdered plant material.

Table 4.1: The weight of powdered plant material

Initial weight (beaker)	277.47g
Final weight (beaker + plant powder)	387.73g
Weight of plant powder	110.26g

Interpretation: The weight of the powdered plant material in the beaker before the maceration process was 110.26 of powdered plant material and which is ready for maceration process.

4.1.2. The net weight of the plant extract obtained after maceration

After the maceration procedure the weight of the plant material is reduced and the table shows the final weight of the leaf extract.

Table 4.2: The total weight of ethanolic grape seed extract obtained as a result of the complete extraction procedure

Initial weight (petri dish)	70.60g
Final weight (petri dish + extract)	84.31g
Weight of extract	13.70g

Interpretation: A total of 13.70g plant extract was produced as a result of maceration and subsequent drying of the ethanolic extract of grape seed that was used in carrying out subsequent experiments.

4.1.3. Calculation of the percentage yield of (*GSE*)

Extract yield (%) = (W_1 x 100) / W_2

Where, W_1 = Net weight of extract after maceration (g)

W_2 = Total weight of powder taken for extraction (g)

Percentage Yield of extract = (13.709/110.26) *100

= 12.43%

Interpretation: The total weight of the extract after maceration was found to be 13.70g from 110.26g powder plant material and therefore, the percentage of yield of *GSE* was 12.43%.

4.2. Determination of percentage yield of the green tea extract (*GTE*)

4.2.1. The total weight of powdered plant material

The table shows the initial weight of the powder along with the beaker and the total weight of the powdered plant material.

Table 4.3: The weight of powdered plant material

Initial weight (beaker)	249.8g
Final weight (beaker + plant powder)	497.5g
Weight of plant powder	247.7g

Interpretation: The weight of the powdered plant material in the beaker before the maceration process was 247.7g of powdered plant material and which was ready for maceration process.

4.2.2. The net weight of the plant extract obtained after maceration

After the maceration procedure the weight of the plant material is reduced and the table shows the final weight of the leaf extract.

Table 4.4: The total weight of ethanolic green tea extract obtained as a result of the complete extraction procedure

Initial weight (petri dish)	68.89g
Final weight (petri dish + extract)	126.91g
Weight of extract	58.01g

Interpretation: A total of 58.01g plant extracts was produced as a result of maceration and subsequent drying of the ethanolic extract of green tea that was used in carrying out subsequent experiments.

4.2.3. Calculation of the percentage yield of the green tea extract (*GTE*)

Extract yield (%) = (W_1 x 100) / W_2

Where, W_1 = Net weight of extract after maceration (g)

W_2 = Total weight of powder taken for extraction (g)

Percentage Yield of extract = (58.016 x 100) / 247.7

= 23.42%

Interpretation: The total weight of the extract after maceration was found to be 58.01g from 247.7g of powdered plant material and therefore, the % yield of *GTE* was 23.42%

4.3 Phytochemical Screening

Table 4.5: Phytochemical screening of *GSE*

Class of compound	**Results**
1. Alkaloid	---
2. Flavonoid	++
3. Phenols/Phenolic compound	+
4. Glycoside	-
5. Tannins	+++
6. Carbohydrate	++
7. Phytosterol	+

8. Resins	+
9. Steroids	+
10. Saponin	+
(+) means presence in a single method test, (++) means presence experimented in two methods, (+++) means presence experimented in three methods, and (-) means absence	

Interpretation: The phytochemical screening of *GSE* showed the presence of flavonoids, phenolic compounds, tannins, carbohydrates, phytosterol, resins, steroids and saponin whilst showing the absence of alkaloid and glycosides.

Table 4.6: Phytochemical screening of *GTE*

Class of compound	**Results**
1. Alkaloid	---
2. Flavonoid	++
3. Phenols/Phenolic compound	+
4. Glycoside	-
5. Tannins	++
6. Carbohydrate	++
7. Phytosterol	+
8. Resins	+
9. Steroids	+
10. Saponin	-
(+) means presence in a single method test, (++) means presence experimented in two methods, (+++) means presence experimented in three methods, and (-) means absence	

Interpretation: The phytochemical screening of *GTE* showed the presence of flavonoids, phenolic compounds, tannins, carbohydrates, phytosterol, resins and steroids whilst showing the absence of alkaloid, saponin and glycosides.

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

4.4.1. Table of DPPH free radical scavenging activity of 1:1 mixture of *GTE and GSE*

The concentration and the absorbance values of sample (1:1 mixture of *GST* and *GSE)* and standard (AA) are shown in the **Table 4.7** below.

Table 4.7: DPPH free radical scavenging activity data or values (Absorbance vs. Concentration)

Concentration (µg/mL)	**Absorbance of Standard (AA) at 517nm (Mean ± SDV)**	**Absorbance of Sample *(GST and GSE)* at 517nm (Mean ± SDV)**
12.5	0.122 ± 0.07	0.251 ± 0.08
25	0.076 ± 0.055	0.22 ± 0.087
50	0.070 ± 0.048	0.032 ± 0.001
100	0.045 ± 0.024	0.019 ± 0.001
200	0.030 ± 0.01	0.02 ± 0.001
400	0.030 ± 0.015	0.018 ± 0.001
800	0.029 ± 0.009	0.018± 0.009
1200	0.027 ± 0.019	0.016 ± 0.001

The **Table 4.7** above shows the absorbance values of the sample and standard corresponding to their respective concentrations at 517nm. The standard deviations are also stated. The concentrations are ranging from 12.5µg/mL to 1200µg/mL.

4.4.2. Graph of Absorbance vs. Concentration for 1:1 mixture of *GSE* and *GTE* and Ascorbic Acid (AA) in DPPH free radical scavenging assay

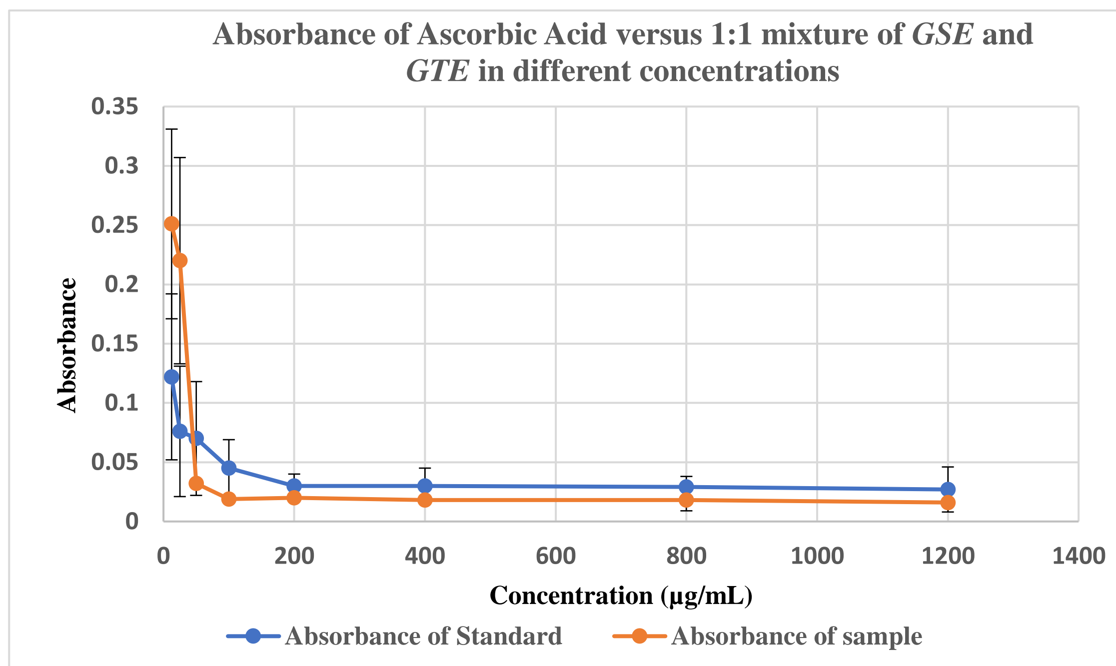


Figure 4.1: Graph of Absorbance vs. Concentration (µg/mL) for ascorbic acid (AA) and 1:1 mixture of *GSE* and *GTE* in DPPH free radical scavenging assay.

Interpretation: From the **Table 4.7** and **Figure 4.1** above, it was observed that with an increase in concentration of 1:1 mixture of *GSE* and *GTE* from 12.5– 1200µg/mL, the absorbance increased gradually in comparison to that of ascorbic acid; thus, implying that the steeper slope i.e. sample (red) had a higher inhibition of DPPH free radical scavengers than the less steep slope (blue) of ascorbic acid standard.

4.4.3. Table of percentage of inhibitory activity of ascorbic acid versus 1:1 mixture of *GSE* and *GTE*

The table next page shows the percentage of inhibition of DPPH free radical scavengers by ethanolic extract of 1:1 mixture of grape seed and green tea.

Table 4.8: % Inhibition of DPPH free-radical scavengers by dried extract of 1:1 mixture of *GSE* and *GTE* with respect to standard AA

Concentration (µg/mL)	% Inhibition (AA) (Mean ± SDV)	% Inhibition (GSE and GTE) (Mean ± SDV)
12.5	72.76 ± 10.219	43.97 ± 13.219
25	83.03 ± 8.219	50.89 ± 12.219
50	84.37 ± 6.219	92.85 ± 0.2
100	89.95 ± 5.109	95.75 ± 0.1
200	93.30 ± 6.095	95.53 ± 0.224
400	93.30 ± 6.819	95.98 ± 0.321
800	93.52 ± 6.219	95.98 ± 0.254
1200	93.97 ± 6.109	96.42 ± 0.855

Interpretation: For concentrations 12.5 to200 µg/mL the % inhibition of free radical scavengers by ascorbic acid and the ethanolic extract of 1:1 mixture *GSE* and *GTE* gradually increased. However, from 200 to 1200 µg/mL, the values for both the standard and sample remained nearly constant. The highest free radical scavenging activity for the sample was found to be 96.42% at 1200µg/mL.

4.4.4. Graph of percentage of inhibitory activity of Ascorbic Acid versus 1:1 *GSE* and *GTE* mixture with corresponding concentrations and their standard deviations

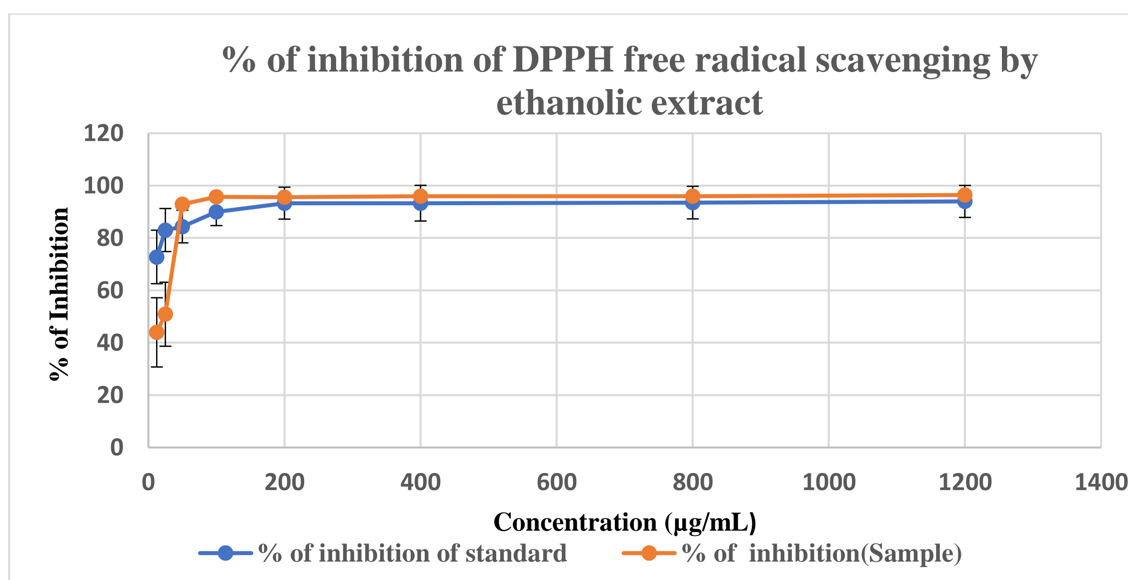


Figure 4.2: % inhibition of DPPH free radical scavengers by 1:1 mixture of *GSE* and *GTE* (red) and standard ascorbic acid (blue) with their standard deviations.

Interpretation: The less-steeper slope (blue) of sample indicates that as its concentration was increased, the % inhibitory activity has also increased from concentration ranging from 12.5 to 50µg/mL. whereas the increase was not significant from concentration 100 to 1200µg/mL. In conclusions, it can be said that with increasing concentrations, the percentage of inhibition did not increase proportionally.

4.4.5. Determination of the IC_{50} of 1:1 mixture of *GSE* and *GTE* and standard ascorbic acid (AA)

Table 4.9: IC_{50} values of 1:1 mixture of *GSE* and *GTE* against standard ascorbic acid

Name of the sample and standard	IC_{50}
1:1 mixture of *GSE* and *GTE* (sample)	75.86 µg/mL
Ascorbic acid (standard)	84.78 µg/mL

Interpretation: The IC_{50} value of dried extract of 1:1 mixture of *GSE* and *GTE* signifies that a much lower concentration of 75.86 µg/mL is required for inhibiting 50% of all DPPH free radical scavengers, whilst a slightly larger concentration of 84.78 µg/mL is required to achieve the same inhibitory effect for ascorbic acid. Thus, the IC_{50} value of the sample is comparable to that of standard. Therefore, it can be said that the plant extract used for this study has exhibited satisfactory amount of anti-oxidant potential.

4.5. Determination of total phenolic content (TPC)

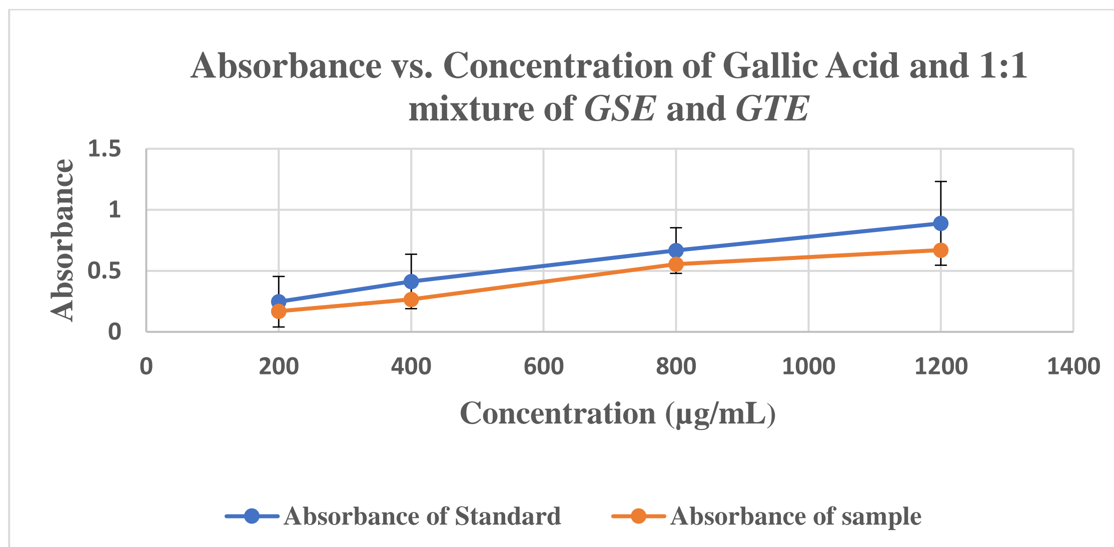


Figure 4.3: Graph of Absorbance vs. Concentration (µg/mL) for Gallic acid and 1:1 mixture of *GSE* and *GTE* with their standard deviations.

4.5.1. Calibration curve of gallic acid

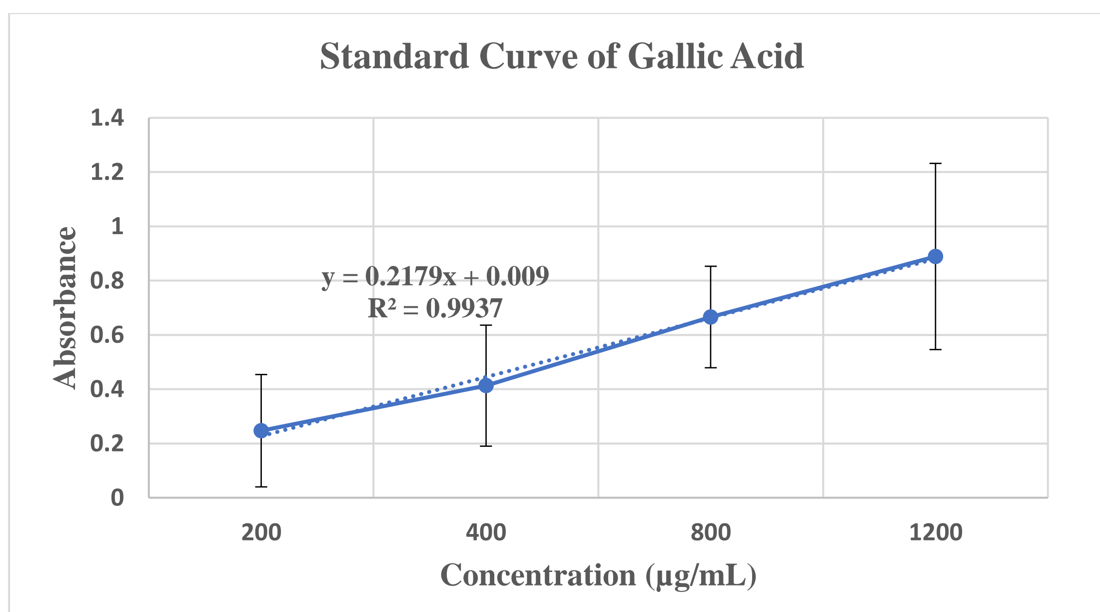


Figure 4.4: Calibration curve of standard gallic acid (GA) at 765nm for determining TPC in 1:1 mixture of *GSE* and *GTE* with standard deviation.

4.5.2. Table of total phenolic content in 1:1 mixture of *GSE* and *GTE*

Table 4.10: Total phenolic content of 1:1 mixture of *GSE* and *GTE* represented as gallic acid equivalent (GAE)

Concentration of sample (µg/mL)	TPC (GAE) (Mean ± SDV)
200	6.45 ± 0.207
400	29.23 ± 0.223
800	96.15 ± 0.187
1200	122.87 ± 0.343
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It is observed that as the concentration of sample was increased from 200 to 1200 µg/mL, the total phenolic content also increased from 6.45 to 122.87mg of gallic acid per gram of dried extract. Therefore, it indicates that with increased in total phenolic content, its antioxidant activity also increased indicating a positive correlation.

4.6. Determination of total flavonoid content (TFC)

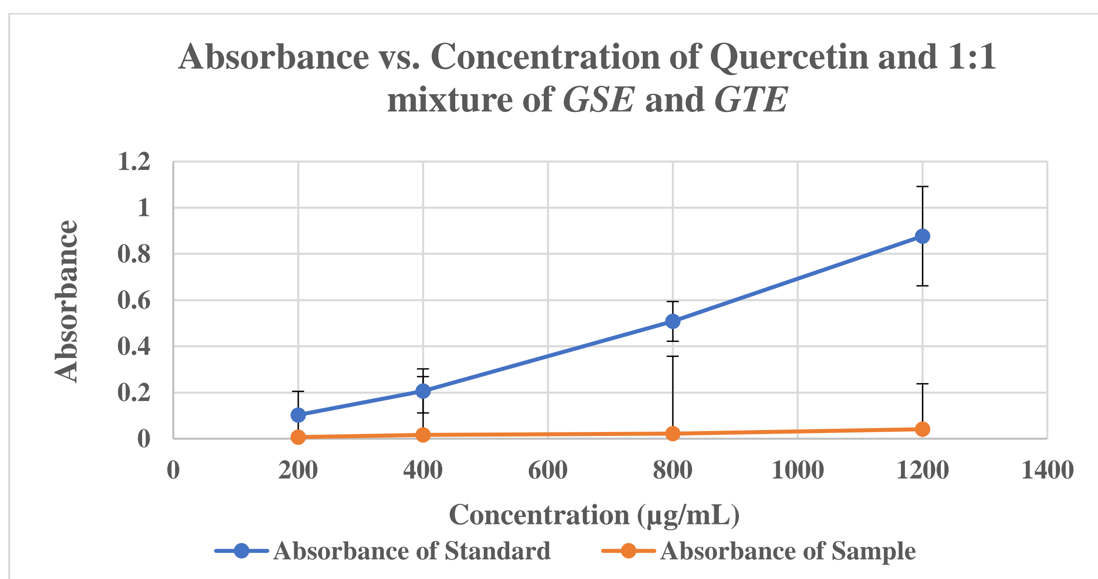


Figure 4.5: Graph of Absorbance vs. Concentration (µg/mL) for Quercetin and 1:1 mixture of *GSE* and *GTE* with standard deviation.

Interpretation: there is no evident positive or negative correlation between the two graphs. The standard shows with increasing concentration, the absorbance is increasing. On the other hand, the sample's absorbance is also increasing with the concentration but the increase is very little and not significant.

The figure shows the calibration curve of standard quercetin for the determination of total flavonoid content.

4.6.1. Calibration curve of quercetin

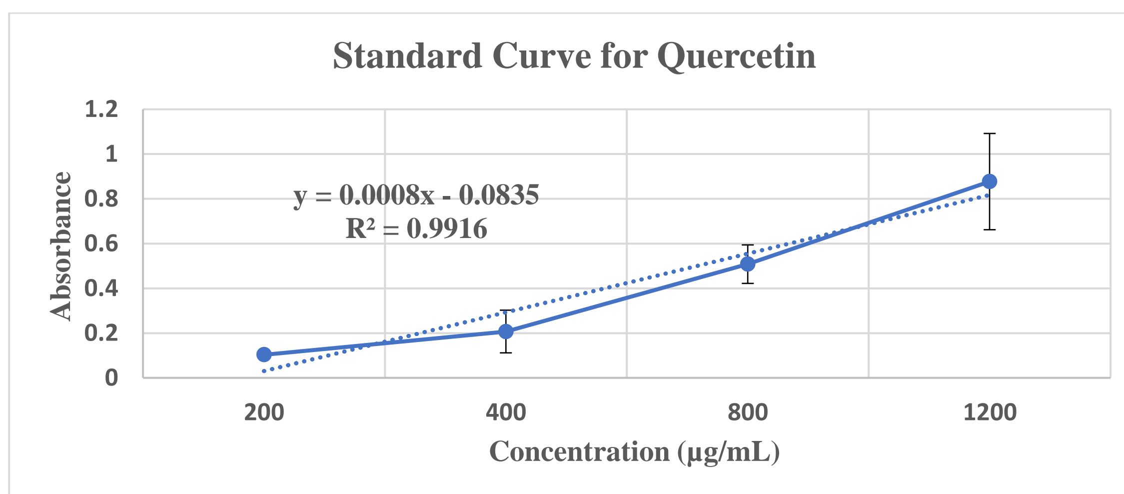


Figure 4.6: Calibration curve of standard quercetin (at 415nm) for determining TFC in 1:1 mixture of *GSE* and *GTE* with their standard deviation.

4.6.2. Table of total flavonoid content (TFC) in 1:1 mixture of *GSE* and *GTE*

Table 4.11: Total flavonoid content of *GSE* and *GTE* combined represented as QE

Concentration of sample (µg/mL)	**TFC (QE) (Mean ± SDV)**
200	15.77 ± 1.098
400	17.34 ± 0.453
800	18.38 ± 0.335
1200	21.69 ± 0.897
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It is observed that as the concentration of sample was increased from 200–1200ug/mL, the total flavonoid content also increased from 15.77 to 21.69 mg of quercetin per gram of dried extract. Therefore, it indicates that there is a positive correlation between total flavonoid content of 1:1 mixture of *GSE* and *GTE* and its antioxidant activity.

4.7. Determination of total antioxidant capacity (TAC)

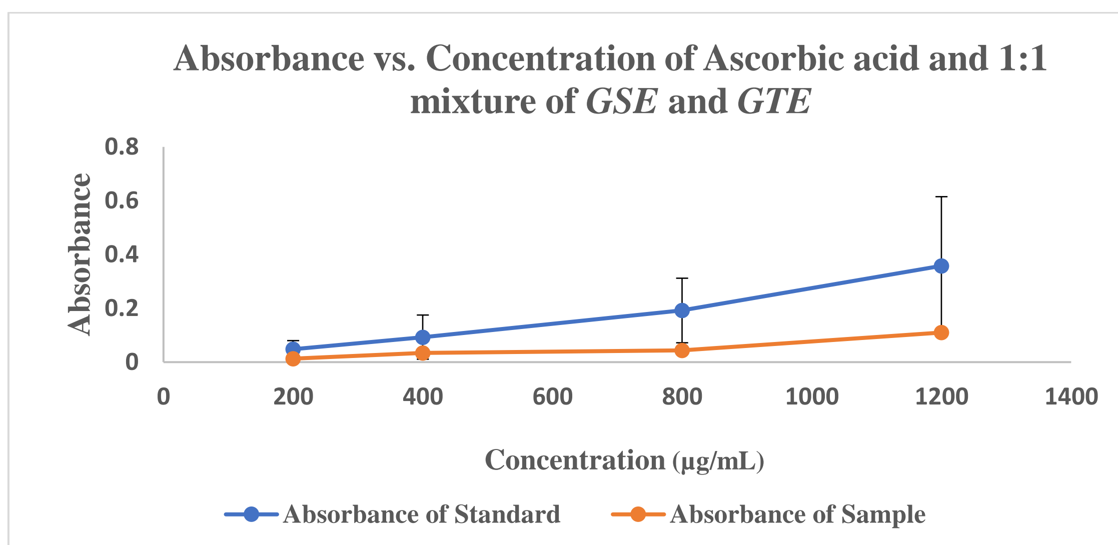


Figure 4.7: Graph of Absorbance vs. Concentration (µg/mL) for Ascorbic acid and 1:1 mixture of *GSE* and *GTE* with standard deviation.

The figure shows the calibration curve of ascorbic acid to determine total antioxidant capacity.

4.7.1. Calibration curve of ascorbic acid (AA)

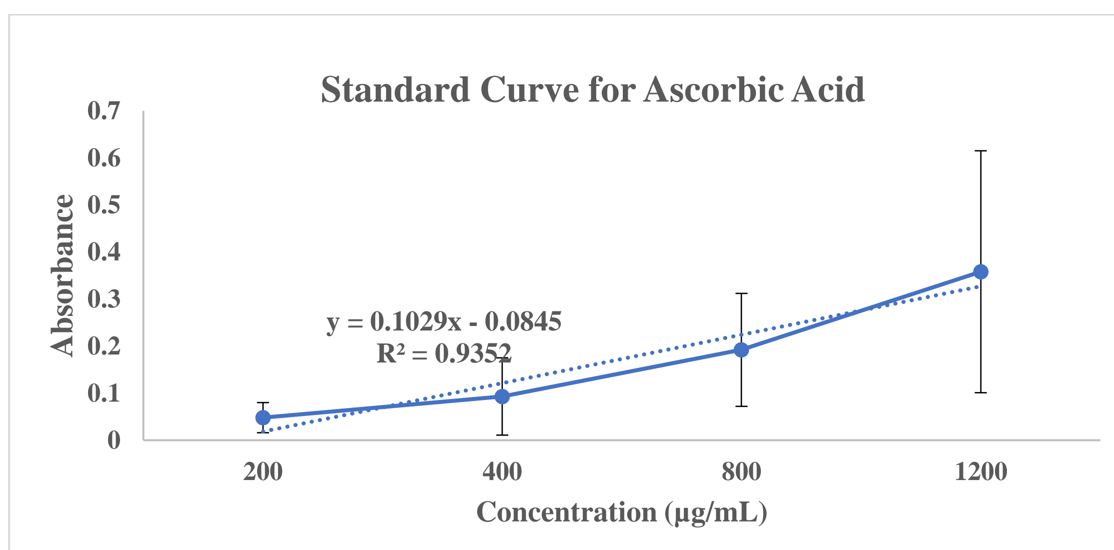


Figure 4.8: Calibration curve of ascorbic acid (AA) at 695nm for determining TAC in 1:1 mixture of *GSE* and *GTE* with standard deviation.

Interpretation: From the graph above, it can be inferred that with the increase in concentration, the absorbance also increased denoting a positive correlation. There are significant standard deviations. The most prominent standard deviation is observed at concentration 1200µg/mL followed by 800µg/mL, 400µg/mL and 200µg/mL. the value for R^2 is also 0.9352 which denotes a good result and linearity.

4.7.2. Table of total antioxidant capacity (TAC) in 1:1 mixture of *GSE* and *GTE*

Table 4.12: Total antioxidant capacity of sample is represented as AAE

Concentration of sample (µg/mL)	TAC (AAE) (Mean ± SDV)
200	18.35 ± 0.332
400	28.11 ± 0.421
800	32.76 ± 0.500
1200	63.43 ± 0.657
The values are the average triplicates of experiments and are represented as mean ± standard deviation	

Interpretation: It is observed that as the concentration of 1:1 mixture of *GSE* and *GTE* was increased from 200µg/mL to 1200µg/mL, the total antioxidant capacity also increased from 18.35 to 63.43 mg of ascorbic acid per gram of dried extract. This indicates therefore, that a positive correlation exists between total antioxidant capacity of the sample and its antioxidant activity.

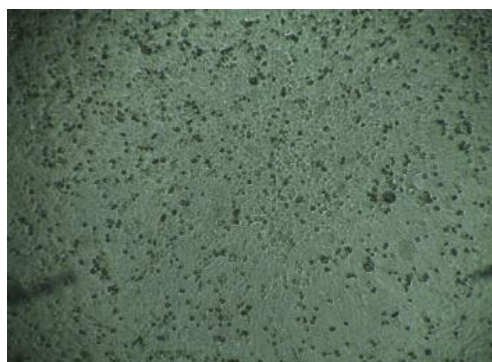
4.8. *In vitro* cytotoxic Activity of ethanolic extract of 1:1 mixture of *GSE* and *GTE* by MTT assay

The cytotoxic activity of 1:1 mixture of *GSE* and *GTE* was performed by MTT assay on HeLa cell line. Different concentration (0.5mg/mL, 1.0mg/mL, 1.5mg/mL, 2.0mg/mL and 2.5mg/mL) of the ethanolic extract were used to analyze the cytotoxic activity. 2% DMSO in DMEM medium was used as negative control. Absorbance was observed for each concentration. Image of cell viability was taken which are provided in **Figure no. 4.9.** The results are given in the **Table 4.10** below:

Table 4.13: Survival of HeLa cells in different concentrations of 1:1 mixture of *GSE* and *GTE* in ethanol along with their % of cell growth inhibition and IC_{50}.

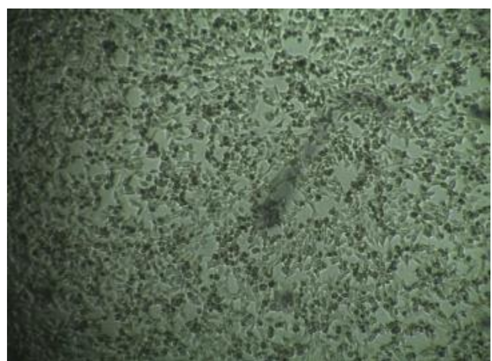
Sample	Survival of HeLa cells (%)	% of cell growth inhibition	IC_{50} (mg/mL)
2% DMSO (negative control)	100	0	8.1
0.5mg/mL	95.51	4.49	
1.0mg/mL	90.66	9.34	
1.5 mg/mL	81.29	18.71	
2.0 mg/mL	68.43	31.57	
2.5 mg/mL	62.03	37.97	

Interpretation: According to the percentage of the survival of the HeLa cells that was observed through trinocular microscope, it is evident that highest cell cytotoxicity was observed on HeLa cell line at 2.5 mg/mL and moderate cytotoxicity at 2.0mg/mL concentration of sample extract. As only 62.03% cells were alive at 2.5mg/mL concentration, it depicts a satisfactory cytotoxic potential. On the other hand, at lower concentrations of 0.5 mg/mL and 1 mg/mL, extract mixture showed very weak cytotoxic activity as the percentage of inhibition was 4.49% and 9.34% respectively.



(a) 0.5 mg/mL of plant extract

(b) 1.0 mg/mL of plant extract



(c) 1.5 mg/mL of plant extract

(d) 2.0 mg/mL of plant extract

(e) 2.5 mg/mL of plant extract

(f) 2% DMSO as negative control

Figure 4.9 (a), (b), (c), (d) (e) and (f): Cell viability of ethanolic extract of 1:1 mixture of *GSE* and *GTE* at different extract concentrations of 0.5mg/mL, 1.0mg/mL, 1.5mg/mL, 2.0mg/mL, 2.5mg/mL and 2% DMSO as negative control respectively after incubating for 48 hours.

Interpretation: **Figure 4.9** shows the cell viability of HeLa cells in different concentrations of the extract as well as in the negative control (2% DMSO). In the **figure 4.9(f)** depicts the negative control where only 2% DMSO solution was added. In the **Figure 4.6(a)**, it is seen that the cell viability decreased due to adding leaf extract of 0.5mg/mL concentration; 4.49% cells growth inhibition was observed. The **Figure 4.9(b)** shows slightly less HeLa cell viability alike second picture as leaf extract of 1.0mg/mL concentration was added. In the third picture i.e. **Figure 4.9(c)** 1.5mg/mL concentration of extract was added and a visible cell growth inhibition was observed which was approximately 18.71%. The **Figure 4.9(d)** shows cell viability at 2.0mg/mL plant extract concentration which shows higher cytotoxicity on HeLa cell line. Lastly **Figure 4.9(e)** depicts the 2.5mg/mL concentration of extract killing the maximum number of cells and exhibiting a remarkable cytotoxicity with almost 37.97% of cell growth inhibition.

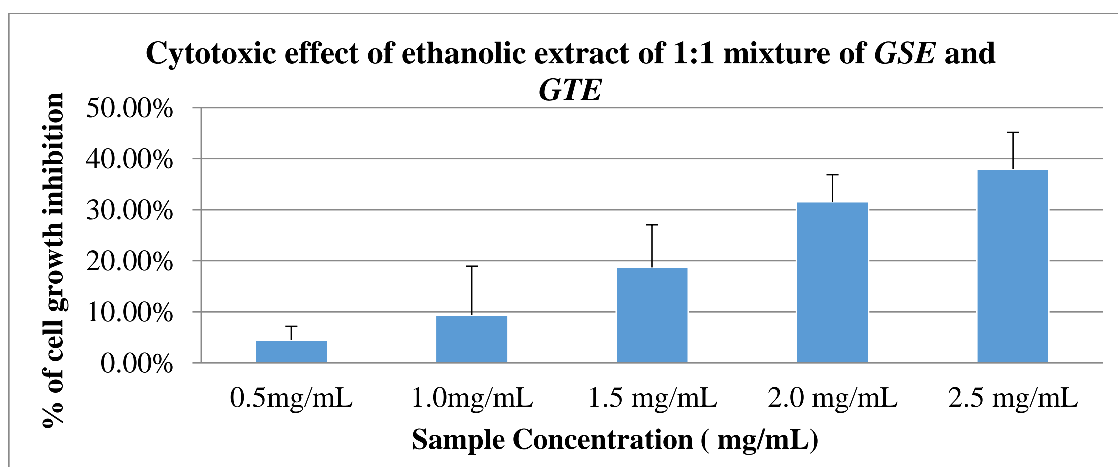


Figure 4.10: Cytotoxic effect of ethanolic extract of 1:1 mixture of *GSE* and *GTE* with their standard deviation.

Interpretation: The **Figure 4.10** is the graphical representation of the percentage of cell growth inhibition versus the respective concentrations of plant extract. For each concentration, the result was calculated in triplicate form and therefore they came with a standard deviation which is also provided on the bar against each concentration. From the bar chart representation, it is very much evident that the lowest percentage of cell growth inhibition was observed at 0.5mg/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 FIVE

DISCUSSION

Chapter 5: Discussion

This study was conducted with a mixture of 1:1 ratio of grape seed and green tea ethanolic extract. For the grape seed, a total of 110.26g of powder was soaked in ethanol and 13.70g of extract was obtained after the extraction process followed by evaporation and drying. This gave a yield of around 12.43% depicting a poor yield value. On the other hand, 247.7g of green tea powder was soaked in ethanol. After the process of evaporation and drying, an extract of 58.05g of extract was obtained, giving a yield value of 23.42% which is almost double of that obtained from grape seed. After obtaining the crude extract various, they were separately screened and then combined in 1:1 ratio mixture in various experiments to get a better understanding of its properties.

Firstly, phytochemical screening was carried out separately using ethanolic *GSE* and *GTE*. The two results manifest the presence of various chemical constituents. Prior to some qualitative tests and experiments, it was found that the *GSE* exhibited presence of flavonoids, phenolic compounds, tannins, carbohydrates, phytosterol, resins, steroids and saponin whilst showing the absence of alkaloid and glycosides. The phytochemical screening of *GTE* showed the presence of flavonoids, phenolic compounds, tannins, carbohydrates, phytosterol, resins and steroids whilst showing the absence of alkaloid, saponin and glycosides. The presence of these components gave us a chance to comprehend and predict the activities that may be found. For an example, the presence of tannin in the plant extract generally indicates anticancer activity (M. T. Lee et al., 2017). Later, the cytotoxicity test on HeLa cell line proved that weak cytotoxic activity was present. Thus, it left us a scope to estimate the anticancer property. In addition, presence of flavonoids, phenols are mainly responsible for antioxidant potential (Flora, 2009).

Then to understand the antioxidant properties of the 1:1 mixture of *GSE* and *GTE*, several *in-vitro* antioxidant tests were carried out such as DPPH free radical scavenging assay, Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Total Antioxidant Capacity (TAC) tests and the findings showed that mixture exhibited moderate antioxidant properties. However specially DPPH free radical scavenging assay showed that 96.42% of inhibition of free radicals are evident for the highest concentration of 1200µg/mL concentration of the green tea and grape seed mixture extract. The IC_{50} was found to be 75.86µg/mL for the plant extract, whereas the IC_{50} was found to be 84.78µg/mL for the standard ascorbic acid which indicated that extract possessed higher antioxidant potential

than the standard ascorbic acid. The result infers that relatively, less amount of sample extract is needed to inhibit 50% of the free radical reaction while the same inhibition can be attained with slightly higher amount of standard ascorbic acid. Thus, highest antioxidant activity was shown by the extract mixture in DPPH free radical scavenging assay. L-ascorbic acid was taken as the standard for DPPH and TAC tests. For Total Flavonoid Content, quercetin and for Total Phenolic Content, gallic acid was taken as standard to compare the values of sample extract. The total phenolic content was found to be 122.87 mg of gallic acid equivalent (GAE)/g. Then total flavonoid content was found to be 21.69 mg of quercetin equivalent (QE)/g and the total antioxidant capacity was found to be 63.43 mg of ascorbic acid equivalent (AAE)/g of crude extract for the concentration of 1200µg/mL. The second highest anti-oxidant activity was evident by TPC method. In a study, seven phenolics in grape seed and green tea extracts were identified separately (not mixture) using methanol as a solvent exhibiting TPC that ranged from 15.38 to 1158.49 mg of chlorogenic acid equivalents/100g of grape seed extract and 18.3 to 1087.02 mg of chlorogenic acid equivalents /100g of green tea extract determined by the Folin-Ciocalteu method (Rababah et al., 2004). When comparing the result of our study i.e. the mixture of 1:1 ratio of *GSE* and *GTE* exhibited higher total phenolic content (TPC) in ethanolic solvent than methanolic solvent using Gallic Acid Equivalents (GAE). Individually the grape seed and green tea in methanol showed lower phenolic content than when they were combined in the 1:1 ratio. This difference may be due to change in solvent, the calculation for the reported study was done in chlorogenic acid equivalents/g and whereas our study calculation was in GAE/g and also may be due to some antagonistic effect that reduces out the total antioxidant potential when the two extracts are combined. In two anti-oxidant assays such as DPPH and TPC, extract mixture showed strong anti-oxidant potential. Whereas other methods such as TAC and TFC showed weak antioxidant potential. Therefore, overall antioxidant potential was moderate.

Anticancer activities were investigated using *in vitro* cytotoxicity test known as MTT assay on cervical cancer cell line (HeLa) where different concentrations of plant extracts were used to determine the cell viability. 2% DMSO was used as the negative control and percentage of cell growth inhibition was determined. From the images it was clear that the mortality of the cells increased with the increase in plant extract in it. The population started to decrease as the concentration of the plant extract started to increase in the culture. The highest percentage of cell growth inhibition of 37.97% was seen at 2.5mg/mL

concentration of 1:1 mixture of grape seed and green tea extract in ethanol giving IC_{50} value of 8.1 mg/mL. This result indicated that the plant mixture exhibited weak cytotoxic activity. One of the literature reviews stated that grape extracts mixed with decaffeinated green tea extracts in the ratio of 25:1 in ethanol synergistically inhibited tNOX activity from human cervical carcinoma (HeLa) cells and cancer cell growth (Morré & Morré, 2006) indicating a strong cytotoxic potential. Furthermore, in another study the effect of the mixture of grape seed and green tea on radiation induced immune suppression in rats was examined. The ratio of *GSE* and *GTE* was 1:2 in ethanol and the investigation was conducted on about 5 groups of rats with one of them being control. The results depicted that the groups treated with the *GSE* and *GTE* mixture depicted an increase in concentrations of immune cells such as CD4 and CD8 (El-Desouky et al., 2017). The level of pro inflammatory cytokines Tumor Necrosis Factor-α and C-Reactive Protein raised up after γ-irradiation and expressively lessened by mixture administration indicating strong anti-cancer properties (El-Desouky et al., 2017). The results obtained from the literature reviews indicate that grape seed and green tea extract in ethanol when combined in other ratios such as *GSE* and *GTE* being 25:1 or 2:1 exhibit stronger cytotoxic potential than 1:1 ratio used for our research which is denoted by the poor IC_{50} value of 8.1mg/mL. The discrepancies may not only be due to the ratio but also due to employment of different *in-vitro* method and in addition, *in-vivo* testing methods were utilized using mice models which have many different physiologic factors affecting the result.

Lastly, it can be concluded that ethanolic extract of 1:1 mixture of *GSE* and *GTE* has weak cytotoxic activity and moderate/mild antioxidant potential. Thus, it is not highly recommended as anticancer agent to treat cervical cancer but most definitely can be used to treat oxidative degenerative diseases like Alzheimer’s disease and Parkinson’s disease.

CHAPTER SIX

CONCLUSION

Chapter 6: Conclusion

This study was conducted with a mixture of 1:1 ratio of grape seed and green tea ethanolic extract. Firstly, phytochemical screening revealed that the *GSE* exhibited presence of flavonoids, phenolic compounds, tannins, carbohydrates, phytosterol, resins, steroids and saponin whilst showing the absence of alkaloid and glycosides. Whereas, *GTE* showed the presence of flavonoids, phenolic compounds, tannins, carbohydrates, phytosterol, resins and steroids whilst showing the absence of alkaloid, saponin and glycosides. Amongst several *in-vitro* antioxidant assays used, DPPH free radical scavenging assay gave IC_{50} value of 75.86 µg/mL for plant extract compared to 84.78 µg/mL for standard ascorbic acid. Total Phenolic Content (TPC) and DPPH assay exhibited strong anti-oxidant potential but Total Flavonoid Content (TFC) and Total Antioxidant Capacity (TAC) tests showed poor antioxidant potential. Therefore overall, the anti-oxidant potential was moderate. Anticancer activities examined using MTT assay denoted IC_{50} value of 8.1 mg/mL which indicated weak cytotoxic potential. To conclude, it can be stated that ethanolic extract of 1:1 mixture of grape seed and green tea has weak cytotoxic activity and moderate antioxidant potential. Thus, it can be used treat oxidative degenerative diseases but cannot be used effectively for cervical cancer until further purification of compounds from this extract is done. This shows a promising future to investigate more about 1:1 mixture of *GSE* and *GTE* in the ground of new drug discovery.

Future Prospects:

- Other cancerous cell lines can be used to carry out the cytotoxicity investigation such as, prostate cancer, breast cancer etc. cell line as no other cell lines were used previously to study cytotoxic potential.
- More detailed isolation of the bioactive compounds can be carried out to investigate the presence of definite structural bioactive compounds as well as their quantitative analysis using high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) etc.
- This study only focuses on the ethanolic extract of *GSE and GTE.* In future other combinations of ratio along with other extraction solvent except ethanol can also provide new scopes to reveal more about this plant's bioactivity.
- They can be used for decreasing the harmful effects of smoking by incorporating a filter made of *GSE* and *GTE.*

CHAPTER SEVEN

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