Investigation of *in vitro* Antioxidant and Cytotoxic Potential of Triphala Methanol Extract in Different Ratios

By

Shifat Sehrin Khan Upoma

16146036

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

Department of Pharmacy

Brac University

February 2020

© 2020. Brac University
All rights reserved.

Declaration

It is hereby declared that

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

Student's Full Name & Signature:

Shifat Sehrin Khan Upoma

16146036

Approval

The thesis titled "Investigation of in vitro Antioxidant and Cytotoxic Potential of Triphala Methanol Extract in Different Ratios" submitted by Shifat Sehrin Khan Upoma (16146036) of Spring, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on 27th February, 2020.

Examining Committee:

Supervisor:
(Member)

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

Program Coordinator:
(Member)

____
Dr. Hasina Yasmin
Professor, Department of Pharmacy
Brac University

Departmental Head:
(Chair)

____
Professor Dr. Eva Rahman Kabir
Chairperson, Department of Pharmacy
Brac University

Ethics Statement

This study does not involve any animal or human trial.

Abstract

Triphala is comprised of Bibhitaki Amalaki and Haritaki and its methanol extract was chosen for the *In vitro* antioxidant and cytotoxic potential studies. Firstly, in the DPPH test the IC_{50} value was found to be 15.93 µg/ mL, 12.99 µg/ mL and 10.287 µg/ mL for Haritaki, Bibhitaki and Amlaki, and the IC_{50} value for Ascorbic Acid was 12.96 µg/ mL, so Amlaki showed more inhibition. The IC_{50} value for the Triphala ratios 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H) and 1:1:2 (A:B:H) were 11.28 µg/ mL, 7.01 µg/ mL, 8.33 µg/ mL and 11.03 µg/ mL respectively and all the values were lower than that of the standard. Secondly, at 1200 µg/ mL, the total phenolic content was found to be 368.94 mg, 395.09 mg and 260.65 mg for Bibhitaki, Amlaki and Haritaki respectively. Finally, at 1200 µg/ mL, the total antioxidant capacity was found to be 245.32mg, 485.5mg and 559.76mg for Bibhitaki, Amlaki and Haritaki respectively. In N4X4 cell line, IC_{50} value was 13.43mg/ mL and 13.23mg/ mL at 25 mg/ mL for 1:1:1(A:B:H) and 2:1:1 (A:B:H) Triphala ratio. To conclude, it can be inferred that the extracts showed strong antioxidant and moderate cytotoxic potential.

Keywords: Antioxidant; DPPH; Cytotoxicity; N4X4; Triphala

Dedication

Dedicated to my beloved family members, for their unconditional love and support.

Acknowledgement

I would like to start by thanking the Almighty ALLAH for enabling me to carry out this research in good health and enriching me with the knowledge, strength, willpower, dedication, skill and opportunity for finishing this thesis paper satisfactorily.

I would like to give my cordial thanks and immense gratitude and will always be indebted towards my respected supervisor, Dr. Raushanara Akter, Associate Professor, Department of Pharmacy, Brac University, for her valuable time and guidance, motivation and most importantly her kindness. Every time I got derailed during my project, she listened to me patiently, clarified my doubts and encouraged me to go forward. Therefore, she will always remain a source of inspiration and guidance for me.

I am also grateful to Professor Dr. Eva Rahman Kabir, Honourable Chairperson, Department of Pharmacy, Brac University, for giving me the opportunity and inspiration to complete my project work and B. Pharm programme.

I would also like to sincerely thank the laboratory officers for their persistent support and assistance and also the respective laboratory officers from Centre for Advanced Research in Sciences (CARS) of Dhaka University for their direction while conducting the laboratory work of my project.

Moreover, special gratitude to my respected faculty members of Department of Pharmacy, BRAC University, for their motivation and support.

Finally, I would like to express my gratitude towards my family and friends for their love, prayers and support, and for believing in me.

Table of Contents

Declaration ii

Approval iii

Ethics Statement iv

Abstract v

Dedication vi

Acknowledgement vii

List of Tables xiv

List of Figures xvi

List of Acronyms xviii

Chapter 1 1

Introduction 1

1.1 Medicinal plants used for phytotherapy 1

1.2 Overview of Bangladeshi Medicinal Plants 3

1.3 Reactive Oxygen Species: Source and Side-effects 4

1.4 Antioxidants 5

1.4.1 Significance of Antioxidant Potential of Plants *5*

1.5 *In vitro* Antioxidant Assay 7

1.6 Brain Tumour 7

1.6.1 Types of Glioma *7*

1.6.2 Anticancer drugs from Medicinal Plants *7*

1.7 *In vitro* Cytotoxicity Screening 9

1.7.1 MTT (3 - (4,5 – dimethylthiazol-2-yl) - 2, 5 - diphenyltetrazolium bromide) assay *9*

1.8 Selection of *Triphala* for the Study 10

1.9 Rationale of This Study 10

1.10 Aim of This Study 11

1.11 Objectives of This Study 11

1.12 Introduction of the Plant 11

1.12.1 Phyllanthus emblica or Emblica officinalis (Amlaki) 11

1.12.1.1 General Description* *11

1.12.1.2 Traditional Uses *12*

1.12.1.3 Constituents *12*

1.12.1.4 Taxanomical Classification *12*

1.12.2 *Terminalia bellirica* (Bibhitaki) 13

1.12.2.1 General Description *13*

1.12.2.2 Traditional Uses *13*

1.12.2.3 Constituents *13*

1.12.2.4 Taxonomical classification *13*

1.12.3 *Terminalia chebula* (Haritaki) 14

1.12.3.1 General Description *14*

1.12.3.2 Traditional Uses *14*

1.12.3.3 Constituents *14*

1.12.3.4 Taxonomical classification 15

Chapter 2 .... **17**

Literature Review **17**

2.1 Previously Studied Pharmacological Activities of Triphala 17

2.1.1 Triphala as An Antineoplastic Agent 19

2.1.2 Triphala as A Radioprotective Effect 19

2.1.3 Triphala as A Chemoprotective Agent 20

2.1.4 Triphala as A Chemopreventive Agent 20

2.1.5 Antimicrobial Activity 20

2.1.6 Antihyperglycemic Effects 21

2.1.7 Antipyretic and Analgesic Activities 21

2.1.8 Antioxidant Effects 21

Chapter 3 .... **22**

Methodology **22**

3.1 Collection and Authentication 22

3.2 Extraction Procedure 22

3.2.1 Extraction ***22***

3.2.2 Filtration ***22***

3.2.3 Concentration and Drying ***23***

3.3 *In vitro* Antioxidant Assay 23

3.3.1 DPPH (1, 1-diphenyl-2-picryl hydrazyl) Free Radical Scavenging Assay 23

3.3.1.1 Reagents and Chemicals 23

3.3.1.2 Sample and Standard Solution Preparation *24*

3.3.1.3 DPPH solution preparation *24*

3.3.1.4 Experimental Procedure *24*

3.3.2 Determination of Total Phenolic Content (TPC) *25*

3.3.2.1 Reagents and Chemicals *25*

3.3.2.2 Reagent Preparation *25*

3.3.2.3 Sample and Standard Preparation *26*

3.3.2.4 Preparation of Blank *26*

3.3.2.5 Experimental Procedure *26*

3.3.3 Determination of Total Antioxidant Capacity (TAC) *27*

3.3.3.1 Reagents and Chemicals *27*

3.3.3.2 Reagent Preparation *27*

3.3.3.3 Sample and Standard Preparation *28*

3.3.3.4 Preparation of the Blank *28*

3.3.3.5 Experimental Procedure *28*

3.4 Cytotoxicity Study by MTT Assay 29

3.4.1 N4X4 Cell Line *29*

3.4.2 Solution Preparation *29*

3.4.2.1 1% Penicillin–streptomycin Solution *29*

3.4.2.2 10 % Fetal Bovine Serum *29*

3.4.2.3 2% DMSO Solution *30*

3.4.2.4 Trypsin *30*

3.4.2.5 Used Consumables 30

3.4.2.6 Used Instruments 30

3.4.2.7 Celltiter 96 Assay Kit 30

3.4.3 Preparation of the Different Concentrations of Plant Extract 30

3.4.4 Cell Culture 31

3.4.4.1 Preparation of Assay Plates 31

3.4.4.2 Thawing of Cells 31

3.4.4.3 Cell Passage 31

3.4.4.4 Harvesting of Cells 32

3.4.4.5 Counting of Cells 32

3.4.5 Procedure 33

Chapter 4 34

Results 34

4.1 Determination of Percentage Yield of the Plant Extract *Triphala* 34

4.1.1 Total Weight of Powdered Plant Material 34

4.1.2 Calculation of Percentage Yield of the Extract 34

4.2 DPPH (1, 1-diphenyl-2-picryl hydroxyl) Free Radical Scavenging Assay 36

4.2.1 Table of DPPH free Radical Scavenging Activity of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala 36

4.2.2 Graph of Absorbance vs. Concentration of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala in DPPH free radical scavenging assay …………38

4.2.3 Table of Percentage of Inhibition of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and different ratios of Triphala 40

4.2.4 Graph of % Inhibition of DPPH Free Radical by Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala 42

4.2.5 Determination of the IC_{50} of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala 44

4.3 Total Phenolic Content (TPC) Determination 45

4.3.1 Calibration Curve of Gallic Acid at 765 nm 45

4.3.2 Table of total phenolic content in Triphala 45

4.4 Total Antioxidant Capacity Determination 46

4.4.1 Calibration curve of Ascorbic Acid (Standard) at 695 nm 46

4.4.2 Table of Total Antioxidant Capacity (TAC) in Triphala 47

4.5 In vitro Cytotoxicity Test of Triphala by MTT Assay on N4X4 (Human Glioma) Cell Line 47

4.5.1 Cytotoxicity Test on N4X4 Cell Line using 1:1:1(A:B:H) ratio 48

4.5.2 Cytotoxicity Test on N4X4 Cell Line using 2:1:1 (A:B:H)ratio 50

Chapter 5 53

Chapter 6 56

References 58

List of Tables

Table 1: Name of the traditional medicinal plants, their parts used in the treatment of common diseases 2

Table 2: Name of the traditional medicinal plants used in Bangladesh (Ghani, 2003a)........ 3

Table 3: Compounds isolated from medicinal plants with their mechanism of action........ 8

Table 4: Previous studies on pharmacological activities of Triphala (Gupta, 2010)........ 17

Table 5: Reagents and chemicals required to determine DPPH scavenging activity........ 23

Table 7: Reagents and chemicals required to determine total antioxidant activity........ 27

Table 8: % yield of Amlaki, Bibhttaki and Haritaki extracts........ 34

Table 9: Table of DPPH Free Radical Scavenging Assay at 517 nm (Absorbance vs. concentration)........ 36

of ascorbic acid, Bibhitaki, Amlaki and Haritaki........ 36

Table 10: Table of DPPH Free Radical Scavenging Assay at 517 nm (Absorbance vs. concentration) of the 37

different ratios of Triphala 37

Table 11: Table of % Inhibition of DPPH Free Radical by Ascorbic Acid, Bibhitaki, Amlaki and Haritaki 40

Table 12: Table of % Inhibition of DPPH Free Radical by different ratios of Triphala 41

Table 13: IC_{50} values of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala 44

Table 13: Total phenolic content (TPC) of Bibhitaki is marked as gallic acid equivalent (GAE) 45

Table 14: Total antioxidant content (TAC) of Triphala is marked as Ascorbic Acid equivalent (AAE) 47

Table 15: Survival and inhibition of N4X4 cell line in different concentrations along with their IC_{50} value and average absorbance using the 1:1:1 (A:B:H) ratio 48

Table 16: Survival and inhibition of N4X4 cell line in different concentrations along with their IC_{50} value and average absorbance using 2:1:1 (A:B:H) ratio 50

List of Figures

Figure 1: Organs affected by oxidative damage 5

Figure 2: Structures of polyphenols exerting antioxidant effect 6

Figure 3: Structure of MTT and colored formazan product (Riss et al., 2013) 10

Figure 4(a): Phyllanthus emblica or Emblica officinalis (Amlaki) 16

Figure 4(b): Terminalia bellirica (Bibhitaki) 16

Figure 4(c): Terminalia chebula (Haritaki) 16

Figure 7: Hemocytometer 32

Figure 8: Graph of Absorbance vs. Concentration (µg/ mL) for Ascorbic Acid, Bibhitaki, Amlaki and Haritaki in DPPH free radical scavenging assay 38

Figure 9: Graph of Absorbance vs. Concentration (µg/ mL) for different ratios of Triphala in DPPH free radical scavenging assay 39

Figure 10: Graph of % Inhibition of DPPH Free Radical by Ascorbic Acid, Bibhitaki, Amlaki and Haritaki 42

Figure 11: Graph of % Inhibition of DPPH Free Radical by different ratios of Triphala 43

Figure 12: Standard curve of standard gallic acid at 765 nm to determine the TPC in sample 45

Figure 13: Standard curve of ascorbic acid at 695 nm for determining TAC value in methanol extract of Triphala 46

Figure 14: Cell viability of methanol extract of Triphala, 1:1:1(A:B:H) ratio, at different extract concentrations of 0.0025 mg/ mL, 0.025mg/ mL, 0.25 mg/ mL, 2.5 mg/ mL, 25 mg/ mL and 2% DMSO as control respectively after incubating 48 hours in N4X4 cell line 49

Figure 15: Cell viability of methanol extract of Triphala, 2:1:1 (A:B:H) ratio, at different extract concentrations of 0.0025 mg/ mL, 0.025mg/ mL, 0.25 mg/ mL, 2.5 mg/ mL, 25 mg/ mL and 2% DMSO as control respectively after incubating 48 hours in N4X4 cell line 51

Figure 16: Cytotoxic effect of methanol crude extract on N4X4 cell line 52

List of Acronyms

WHO:	World Health Organization
IARC:	International Agency for Research on Cancer
A:	Amlaki
B:	Bibhitaki
H:	Haritaki
AA:	Ascorbic Acid
ROS:	Reactive Oxygen Species
TPC:	Total Phenolic Content
TAC:	Total Antioxidant Capacity
DPPH:	1, 1-Diphenyl-2-Picryl Hydrazyl
FCR:	Folin Ciocalteu Reagent
MET:	Methanol Extract of *Triphala*
GA:	Gallic Acid
LAF:	Laminar Air Flow

Chapter 1

Introduction

1.1 Medicinal plants used for phytotherapy

World Health Organisation (WHO) defines medicinal plants as plants that holds therapeutic properties or compounds useful as therapeutics or those that produce metabolites that act as useful drugs (WHO 2008). Different parts of plants are used in the modern treatment and these different parts of the medicinal plants can be used as different dosage forms, such as decoctions, pastes, powders, juices, infusions etc. Examples of other famous medicines are colchicines from *Colchicum autumnale,* quinine from *Cinchona* spp. and caffeine from *Thea sinensis* (Burbage, 1981). Phytotherapy means the treatment using natural plant-derived medicines and in today's world plant-derived medicines have become a primary source of pharmaceuticals due to their characteristic pharmacological activity on human body and the most useful ones are flavonoids, phenolic compounds, and alkaloids which hardly contains toxins (Devendra, Srinivas, & Solmon, 2012). According to the World Health Organization (WHO), about 80 % of developing countries rely on traditional medicines for their primary health care. (Sofowora, Ogunbodede, & Onayade, 2013). Table 1 illustrates some name of the traditional medicinal plants along with their parts used in the treatment.

Table 1: Name of the traditional medicinal plants, their parts used in the treatment of common diseases

Scientific Name (Family)	Part used	Traditional uses	References
Curcuma longa L	Rhizome	Irritation & itching, inflammation, erythema, burns & sunburn and other skin diseases	Zari, S. T., &Zari, T.A. (2015).
Plumbago zeylanica L.	Root	Paralysis, secondary syphilis, leprosy and ophthalmics	Chopra, R.N., Nayer, & Chopra, I.C. (1992).
Aloe vera L.	Leaf gel	Psoriasis, cold sores, sun burns, microbial infections on skin	Zari, S. T., &Zari, T.A. (2015).
Acanthus ilicifolius L	Root	Leucorrhoea	Hossan et al. (2010).
Holarrhena antidysenterica Wall	Bark	Dysentery, dropsy, fever, diarrhea and intestinal worm infections	Chopra, R.N., Nayer, & Chopra, I.C. (1992).

1.2 Overview of Bangladeshi Medicinal Plants

According to the WHO, about 80 % of population from developing countries rely on Bangladeshi medicinal plants for primary treatment. Furthermore, due to better cultural acceptability, improved compatibility with human body and fewer side effects about 25 % of prescribed drugs come from plants and herbal medicines (Jafor & Obaidullah, 2018). For many years numerous plants have been used to treat health conditions using traditional medicine. Some examples are given in Table 2.

Table 2: Name of the traditional medicinal plants used in Bangladesh (Ghani, 2003a)

Bangali Name	Scientific Name	Parts of plants	Uses
Mehedi	Lawsonia inermis	Leaves, flower	1. Skin disease 2. Pox 3. Burn 4. Dandruff
Thankuni	Cliotoria ternatea	Whole plant	1. Weakness 2. Dermatitis 3. Jaundice 4. Stomach disorder
Arjun	Terminalia arjuna	Bark	1. Heart disease
Halud	Curcuma longa	Rhizomes	1. Blood purification 2. Skin and eye disease 3. Stomachache

Continued

Lajjabati	Mimosa pudica	Whole plant	1. Toothache 2. Convulsion 3. Blood purification 4. Fistula 5. Piles
Nayantara	Catharanthus roseus	Flowers	1. Cancer 2. Diabetes 3. Insomnia 4. Blood pressure

1.3 Reactive Oxygen Species: Source and Side-effects

Aerobic metabolism in the cell is continuously making oxygen-derived species such as hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) and free radicals such as superoxide (O_2), nitric oxide (NO) and hydroxyl (OH). Furthermore, exposure to oxidizing air pollutants, tobacco smoke, and car exhausts lead to the production as well. Even with the body’s antioxidant defense systems to protect against these reactive oxygen species, oxidative damage is imminent. Severe stress can damage DNA, proteins, and lipid causing cell transformation or cell death, thus causing human diseases such as cardiovascular disease and cancer (Helliwell B. and Aruoma, 1993, Helliwell B., 1994, Halliwell B, Gutteridge, 1989).

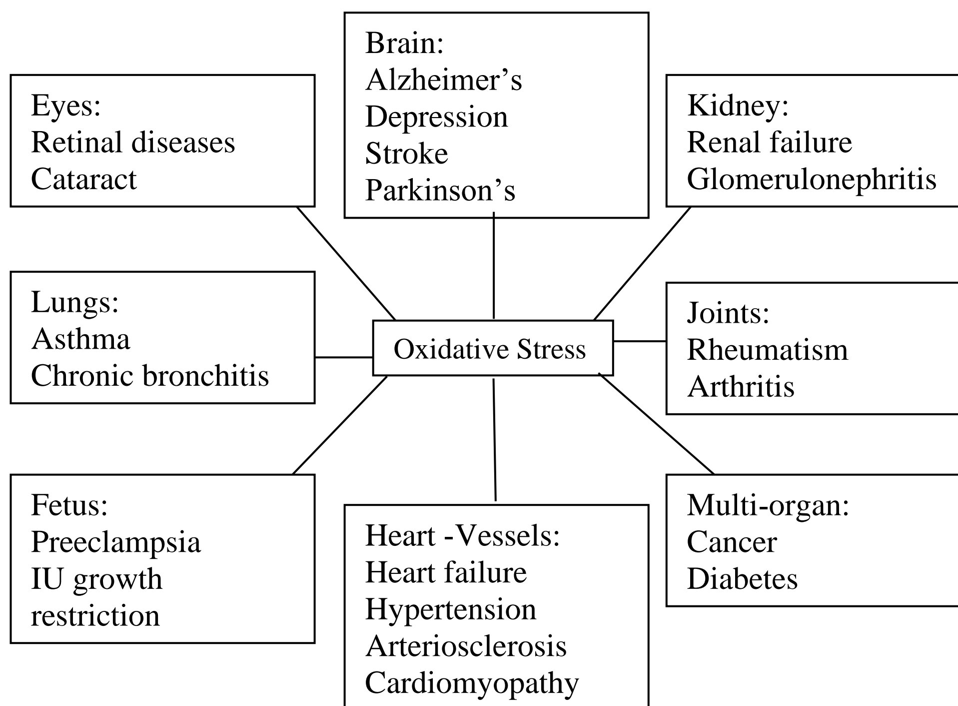

Figure 1: Organs affected by oxidative damage

In the figure 1, it is highlighted that the buildup of oxidative stress caused by reactive oxygen species (ROS) in several organs will destroy the cells and eventually cause diseases.

1.4 Antioxidants

Antioxidants are compounds that considerably reduces the oxidation of a substrate at small concentrations compared to that of an oxidizable substrate (Young, 2001). Natural as well as synthetic antioxidants are used to treat diseases and prevent cellular damage done by ROS or free radicals. The medicinal plants exhibiting antioxidant properties generally contain polyphenols and flavonoid, a polyphenolic chemical, which are considered the main antioxidant compounds.

1.4.1 Significance of Antioxidant Potential of Plants

Plants have an intrinsic ability to generate a wide range of non-enzymatic cancer prevention agents which work to mitigate the oxidative damage stimulated by ROS. Plants promote the production and aggregation of low-weight cancer preventive agents such as

vitamin C, vitamin E, phenolic acids, and high-weight cancer preventive agents such as tannins. These work as reducing agents, free radical scavengers and metal chelators. To prevent the toxic consequences of the free radicals,

plants have effective enzymatic defence systems called catalase (CAT), glutathione peroxidase(GPx), and glutathione reductase (GR) and non-enzymatic defense systems called ascorbic acid, glutathione, lignan, pro-line, stilbene, carotenoids, phenolic acids, flavonoids, tannins etc. Polyphenols and carotenoids are the two major classes of natural antioxidants in our foods and medications. (Tsao, 2010).

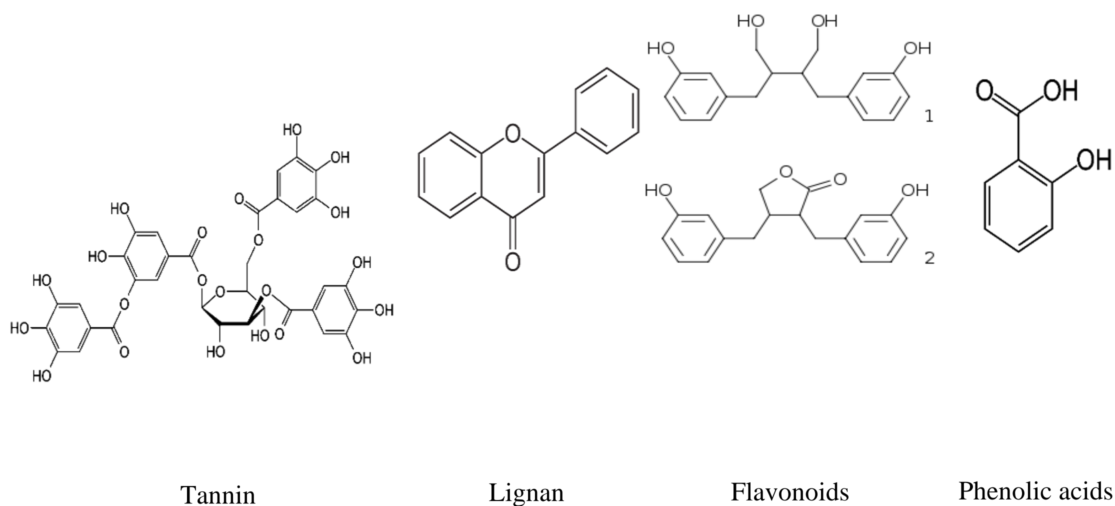

Figure 2: Structures of polyphenols exerting antioxidant effect

1.5 *In vitro* Antioxidant Assay

The use of DPPH, TAC, TPC and TFC assays can determine radical scavenging capacity of various extracts. Compared with other available methods, these methods are more reliable, accurate and cheap. (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos & Hawkins Byrne, 2006). When compared with other procedures for free radical scavenging, DPPH is considered the fastest and most convenient (Alam et al, 2013).

The reaction between DPPH and antioxidants (H-A) is given below:

DPPH + HA: DPPH-H + HA

In the reaction, after the substrate reacts, it turns DPPH (1, 1-diphenyl-2-picrylhydrazyl) yellow from red color. Antioxidants bind to form DPPH-H with the loss of red color and ultimately leading to diminished absorbance. This discoloration indicates antioxidant compound's scavenging activity.

1.6 Brain Tumour

Brain tumor refers to a mixed group of neoplasms that form from the intracranial tissues and abnormal brain cell growth due to mutations. Brain tumors are the second most common cancer in children, and consist around 15-25% of pediatric malignancies, around 40% glioma and around 25% medulloblastomas.

1.6.1 Types of Glioma

The main types of gliomas are Astrocytomas, Oligodendoglioma, Ependymomas and Glioblastoma.

1.6.2 Anticancer drugs from Medicinal Plants

For their basic health needs, about 80 per cent of the total population of third world nations relies solely on plant products (Mans et al., 2000). Researchers noted that herbal medicinal products are considered to be one of the best treatments for cancer and some known

metabolites with anticancer effects are flavonoids, terpenoids, alkaloids, and phenylpropanoids (Kumar et al., 2012). These compounds may be used alone or in combination with other anticancer treatments. These natural compounds, compared to synthetic drugs, are more easily available, cheaper and can be easily administered orally and at the same time have negligible side effects (Seca & Pinto, 2018; Lichota & Gwozdzinski, 2018). Thazin and colleagues mentioned that natural plant compounds are capable of working as both an anticancer agent as well as for restoring sensitivity to chemotherapy. For example, doxorubicin anticancer activity towards resistant MCF-1/DOX cells in vivo is increased by tetrandrine and it is an active alkaloid compound extracted from plants (Aung et al., 2017). The first agents that have been discovered as an anti-cancer agent are vinca alkaloids (Vinblastine and Vincristine) from the periwinkle plant *Catharanthus roseus.* Some examples of anticancer compounds isolated from plants is shown in Table 3.

Table 3: Compounds isolated from medicinal plants with their mechanism of action

Compound	Drug Class	Plant source	Mechanism of action
Vinblastin, vincristine	Vinca alkaloids	*Catharanthus roseus*	Polymerization inhibition of tubulin
Docetaxel	Taxanes	*Taxus baccata*	Microtubule stabilization
Berbamine	Bis-benzylisquinoline	*Berberis amurensis*	Appoptosis
Hypericin	Nipthodianthrone	*Hypricum perforatum*	Hypercinoids inhibition
Etoposide, Tenoposide	Alkaloids	*Catharanthus roseus*	Topoisomerase inhibitor
Irinotecan, Topotecan	Alkaloids	*Catharanthus acuminate*	Topoisomerase inhibitor

Continued

1.7 *In vitro* Cytotoxicity Screening

Cytotoxicity refers to the property of a chemical capable of causing cell death. The introduction of a cytotoxic agent to a normal or cancerous cell will induce necrosis of the cells and if it kills both normal and cancerous cells, then it is a cytotoxic agent, but if it kills only cancerous cell then it can be a prospective anticancer agent. Different methods for evaluating cytotoxic activity have been developed (Adan, Kiraz & Baran) and MTT assay has been shown to be sensitive, quantitative and accurate among all the tests (Senthilraja & Kathiresan, 2015).

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

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay is a type of tetrazolium reduction assay that iis commonly used to find viability of the cells. First MTT substrate is prepared in a solution, then transferred to cultivated cells, and eventually incubated for 1 to 4 hours. The hypothesis shows the amount of formazan generated is proportional to the number of cells that are viable. The measurement is done using a plate reading spectrophotometer where absorbance is taken at 570nm. (Riss et al.,2013). The reaction is shown in Figure 3.

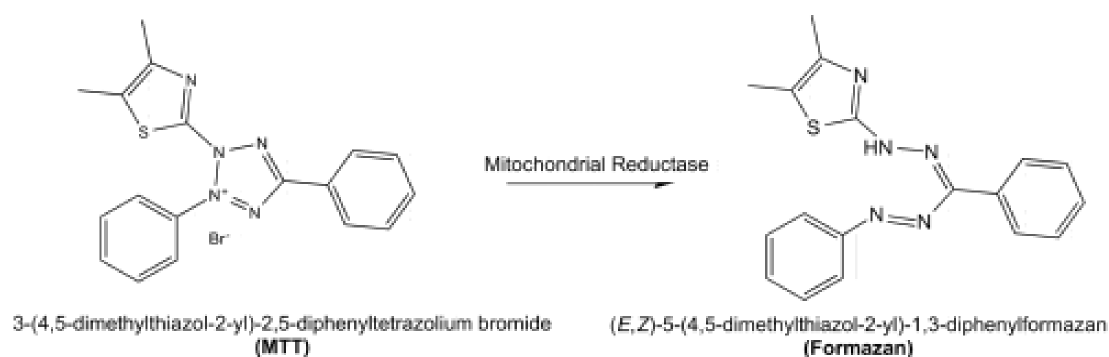

Figure 3: Structure of MTT and colored formazan product (Riss et al., 2013).

1.8 Selection of *Triphala* for the Study

Triphala ['three' (tri) 'fruits' (phala)] is a traditional Ayurvedic herbal formulation consisting of medicinal plant fruits, *Terminalia chebula* (Haritaki), *Terminalia bellirica* (Bibhitaki), and *Phyllanthus emblica* or *Emblica officinalis* (Amlaki). Antioxidant capacity determination of methanol extract was not carried out before in non-equivalent ratios. Methanol extract of *Triphala* showed the highest antioxidant activity among all the solvents. *Triphala* also showed cytotoxic and apoptosis activity against different cancer cell lines, but no study was done using N4X4 cell lines. That is why, methanol extract of *Triphala* is selected for the antioxidant and cytotoxic activity determination with N4X4 cell lines in 1:1:1 (A:B:H) ratio and 2:1:1 (A:B:H) ratio.

1.9 Rationale of This Study

Triphala possess free radical scavenging, antioxidant, anti-inflammatory, antipyretic, analgesic, antibacterial, antimutagenic, wound healing, anticariogenic, adaptogenic, hypoglycaemic, anticancer, chemoprotective, radioprotective and chemopreventive effects. However different ratios of Triphala as a methanol extract was not used before for the assessment of antioxidant activity. In addition, methanol extract of this plant showed strong cytotoxicity against other cancer-cell lines but N4X4 cell lines were not used before to determine the cytotoxic potential. Moreover, non-equivalent ratio of

Triphala was not used to do antioxidant and cytotoxicity test.

1.10 Aim of This Study

The aim of this project is to investigate the *in vitro* antioxidant and cytotoxic activity of methanol extract of *Triphala* (*Terminalia chebula* (Haritaki), *Terminalia bellirica* (Bibhitaki), and *Phyllanthus emblica* or *Emblica officinalis* (Amlaki))

1.11 Objectives of This Study

After studying the literature review from previous findings on Triphala, objectives were made as follows:

- Determination of antioxidant activity using various *in vitro* methods and assessment of free radical scavenging potential comparable to that standard ascorbic acid using the DPPH free radical scavenging method and others
- Determination of cytotoxic activity using MTT assay on N4X4 cell line.

1.12 Introduction of the Plant

Triphala [‘three’ (tri) ‘fruits’ (phala)] is a formulation consisting of *Phyllanthus emblica* or *Emblica officinalis* (Amlaki), *Terminalia chebula* (Haritaki), and *Terminalia bellirica* (Bibhitaki).

1.12.1 Phyllanthus emblica or Emblica officinalis (Amlaki)

1.12.1.1 General Description

Amlaki can be found in India, around the sea-coast districts and on hill slopes of up to 200 meters (Calixto, Santos, Filho & Yunes, 1998) and also found in Burma (Dey KL, 1896).The Emblica officinalis is a medium to small –sized deciduous tree, 8 18 meters high with a thin light grey bark, the leaves are flat, light green with pinnate l eaves. The flowers are greenish yellow, in axillary fascicles, unisexual (Singh and Singh, 2002). Figure 4(a) shows the picture of the fruit.

1.12.1.2 Traditional Uses

Amlaki is traditionally used for many things such as to keep control of blood sugar level, decreasing total cholesterol, eye diseases like cataract and glaucoma. It also has nourishing effect on hair when used as a hair tonic and also on the skin. It is also used for hyperacidity, as a mixture with sugar syrup. (Kumar, 2012)

1.12.1.3 Constituents

EO primarily contains tannins, alkaloids, phenolic compounds, amino acids and carbohydrates. Its fruit juice contains the highest vitamin C (478.56 mg/100 mL). The fruit hincreases nutritional quality in terms of Vitmin C when mixed with other fruits (Shastri and Bhavaprakasha, 1969). Compounds isolated from EO were gallic acid, ellagic acid, 1-O-galloyl-beta-Dglucose, 3, 6-di-O-galloyl-Dglucose, chebulinic acid, quercetin, chebulagic acid, corilagin, 1, 6-di-O - galloyl beta D glucose, 3 Ethylgallic acid (3 ethoxy 4, 5 dihydroxy benzoic acid) and isostrictiniin21. Phyllanthus emblica also contains flavonoids, kaempferol 3 O alpha L (6'' methyl) rhamnopyranoside and kaempferol 3 O alpha L (6''ethyl) rhamnopyranoside22. A new acylated apigenin glucoside (apigenin 7 O (6'' butyryl beta glucopyranoside) was isolated from the methanolic extract of the leaves of Phyllanthus emblica (Nadkarni, 1993).

1.12.1.4 Taxanomical Classification

Kingdom: Plantae

Division: Angiospermae

Class: Dicotyledonae

Order: Geraniales

Family: Euphorbiaceae

Genus: Emblica

Species: Officinalis

1.12.2 *Terminalia bellirica* (Bibhitaki)

1.12.2.1 General Description

It is found in the wild throughout the India, Srilanka, and Southeast Asia. It can be seen at 1200 meters in elevation, in a wide variety of ecologies. It is a large deciduous tree with a buttressed trunk, a thick brownish gray bark, attaining a height of between 20 and 30 meters. The leaves are crowded around the ends of the branches, alternately arranged, margins entire, elliptic to ellipticobovate. The flowers are pale greenish yellow with an offensive odor. The fruits are ovoid grey drupes. (Saroya, 2009, Nadkarni, 2002). Figure 4(b) shows the picture of the fruit.

1.12.2.2 Traditional Uses

Bibhitaki is traditionally used for common cold, pharyngitis and constipation. The bark is midly diuretic and is useful in anaemia and leucoderma. Unripe fruit is a mild laxative and ripe fruit is an astringent. The seeds are used as aphrodisiac and the oil extract from the seed pulp is used in leucoderma and alopecia. (Kumudhavalli, 2010).

1.12.2.3 Constituents

Glucoside (bellericanin), Gallo-tannic acid, Coloring matter, resins and a greenish yellow oil. Ellargic acid, gallic acid, lignans (termilignan and thanni lignan), 7-hydroxy 3’4’ (methylene dioxy) flavone and anolignan B10. Tannins, ellargic acid, ethyl gallate, galloyl glucose and chebulaginic acid, phenyllemblin, β-sitosterol, mannitol, glucose, fructose and rhamnose (Saroya, 2009, Singh, 2006).

1.12.2.4 Taxonomical classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Myrtales

Family: Combretaceae

Genus: Terminalia

Species: bellirica

1.12.3 *Terminalia chebula* (Haritaki)

1.12.3.1 General Description

T. chebula is native in Asia and mainly found in Nepal, Sri Lanka, Myanmar, Bangladesh, Egypt, Iran, Turkey, Pakistan, Yunnan, Tibet, Guangdong, Guangxi province of China. It is a medium-sized, up to 25 m tall, deciduous tree, bark dark brown. Leaves alternate or opposite, thin-coriaceous, ovate or elliptic-obovate, petiole up to 2 cm long. Flowers in axillary 5-7 cm long spikes, simple or sometime branched, yellowish-white and unpleasantly scented. Fruit an obovoid or oblong-ellipsoid drupe, yellow to orange-brown when ripe. (Boer et al., 1995, Fundter et al., 1992, Prakash, 2012). Figure 4(c) shows the picture of the fruit.

1.12.3.2 Traditional Uses

Haritaki is used as an eye tonic, mainly for eye irritations, and also as a hair tonic to reduce greying of hair. It also helps to control vomiting, diarrhea, can increase appetite and also works as a digestive. A liver tonic is also made, which is a jam, for liver problems and also used as a diuretic and treatment of polypus (Chandra, 2012).

1.12.3.3 Constituents

In Terminalia chebula, 33% of the total phytoconstituents are hydrolysable tannins and are responsible for pharmacological activity. These tannins contain phenolic carboxylic acid like gallic acid, ellagic acid, chebulic acid and gallotannins such as 1,6 di-O-galloyl-β-D-glucose, 3,4,6 tri-O-galloyl-βD-glucose, 2,3,4,6 tetra-O-galloyl-β-D-glucose,

1,2,3,4,6 penta-Ogalloyl-β-D-glucose. Ellagitannin such as punacalagin, casurarinin, corilagin and terchebulin and others such as chebulanin, neochebulinic acid, chebulagic acid and chebulinic acid reported in literature (Juang et al., 2004, Han et al., 2006). The tannin content varies with the geological variation. Flavonol glycosides, triterpenoids, coumarin conjugated with gallic acid called chebulin, as well as phenolic compounds were also isolated. (Chottopadhyay and Bhattacharyya, 2006)

1.12.3.4 Taxonomical classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Myrtales

Family: Combretaceae

Genus: Terminalia

Species: chebula

Figure 4(a): Phyllanthus emblica or Emblica officinalis (Amlaki)

("Triphala", 2020)

Figure 4(b): Terminalia bellirica *(Bibhitaki) ("Triphala", 2020)*

Figure 4(c): Terminalia chebula (Haritaki) ("Triphala", 2020)

Chapter 2
Literature Review

2.1 Previously Studied Pharmacological Activities of Triphala

Literature review of *Triphala* and its constituents has been done and it showed that they carry different types of activities as illustrated in table 4.

Table 4: Previous studies on pharmacological activities of Triphala (Gupta, 2010)

Use	Amlaki	Haritaki	Bibhitaki
External Use	• Hair fall • eye diseases • various skin diseases	• Pain • Inflammation • Wounds • various skin diseases	• Pain • Leukoderma • Inflammation • greying of hair
Central Nervous System	• Diseases of sense organs • Enhance memory	• Various neurological disorders • Enhance memory • Eye disease	• Insomnia and various central nervous system (CNS) disorders • Prevents blindness, myopia and eye disorders
Cardio-vascular System	• Cardiac diseases • Blood Disorders	• Cardiac diseases • Oedema • Anaemia and blood discracias	• Bleeding disorders • Haemoptysis
GIT	• Anorexia	• Anorexia	• Anorexia

Continued

	• Impaired digestion • Constipation • Liver disorder • Gastritis • Colic pain • Haemorrhoids • Other gastrointestinal disorders	• Colic pain • Haemorrhoids • Jaundice • Diseases of liver and spleen • Helmentiasis • Abdominal tumours • Abdominal ailments	• Upward movement of gases • Vomiting • Constipation • Haemorrhoids • Helmentiasis
Respiratory System	• Cough, difficulty in breathing • Tuberculosis • Various respiratory disorders	• Rhinitis • Cough • Harshness of voice • Bronchitis • Other respiratory disorders	
Genito-urinary System	• Oligurea • Dysuria • Aphrodisiac • Infertility	• Oligurea • Renal calculi • Urinary disorders • Gout • Aphrodisiac • Infertility	• Aphrodisiac • Improves libido and impotence

2.1.1 Triphala as An Antineoplastic Agent

In last fifty years, chemotherapy has been the foundation of cancer treatment. Most chemotherapeutic medications, however, have toxicity and this has led to the need for an accurate, readily acceptable and inexpensive non-toxic agent. Preclinical studies on Michigan Cancer Foundation-7 (MCF-7) and T47D, which are human breast cancer cells, have shown that Triphala has antineoplastic effects (Kaur et al., 2005; Sandhya et al., 2006; Sandhya & Misha, 2006). Furthermore, it showed effect on PC-3 and DU-145, which are prostate cancer cells (Kaur et al., 2005), Capan-2, BxPC-3 and HPDE-6 cells, which are human pancreatic cancer cells (Shi et al., 20008), Shionogi 115, which are mouse breast cancer cells (Kaur et al., 2005) and barcl-95 transplantable mouse thymic lymphoma (Sandhya et al., 2006) in vitro and repressed the development of Capan-2 (pancreatic tumor) in nude mice (Shi et al., 20008). Research have also shown that Triphala has no cytotoxic effects on normal breast epithelial cells, mouse liver and spleen cells, human peripheral mononuclear blood cells (Sandhya et al., 2006), and HPDE-6 cells (Shi et al., 20008). Studies show that Triphala regulated the cytotoxic effects by increasing intracellular ROS and primarily by inducing apoptosis by the p53 pathway (Sandhya et al., 2006; Sandhya & Misha, 2006; Shi et al., 20008).

2.1.2 Triphala as A Radioprotective Effect

Radiotherapy, while effective in treating cancer causes damage to healthy tissues. The use of radioprotectors that can systemically shield the normal cells from damage is a possible strategy for increasing the therapeutic index, thereby offering tremendous benefit to the patients with cancers. Preclinical studies have shown that Triphala has radioprotective properties when delivered through intraperitoneal (Jagetia et. al, 2002; Jagetia et al.; 2004) and oral routes. When delivered intraperitoneally, 10 mg / kg Triphala was found to be most successful if administered before the irradiation and

caused a reduction in dose. Biochemical tests found that Triphala stabilized levels of xanthine oxidase (XO) and SOD activity in mice's intestine and also minimized damage of DNA in blood leukocytes and splenocytes, suggesting there was inhibition of oxidative damage in cells and organs. (Sandhya et al., 2006)

2.1.3 Triphala as A Chemoprotective Agent

The antifolate class of anticancer drug methotrexate causes severe enterotoxicity which sometimes reduces the therapeutic benefit. Nariya et al researched 1:1:1 and 1:2:4 triphala ratio for Enteroprotective results against methotrexate-induced damage on the intestines of rats by evaluating gross and microscopic harm, testing intestinal permeability to red phenol and biochemical tissue parameters. The authors found that Triphala administration regained the reduced protein level in the brush borders of the intestine, phospholipid, and glutathione amount and decreased Myeloperoxidase (MPO) and XO levels in the mucosa of methotrexate-treated rat's inestine, with greater effects being observed with 1:2:4 mixture. (Nariya et al., 2009)

2.1.4 Triphala as A Chemopreventive Agent

Chemoprevention are non-toxic organic or naturally occurring chemicals, that prevent cancer or reduce the incidence of cancer. Deep, et al showed that Triphala decreased the B(a) P-induced forestomach papillomagenesis in mice and the result depended both on concentration and time. Triphala was shown to be more effective at equal concentration than the individual constituents suggesting that the three constituents act synergestically to mediate the observed chemopreventive effects. (Deep et al., 2005)

2.1.5 Antimicrobial Activity

Reportedly, Triphala and its individual constituents have strong antibacterial activity on different organisms. Triphala's aqueous and ethanol extracts, as well as the

separate components, have shown antibacterial efficacy against isolates acquired from patients diagnosed with the human immunodeficiency virus (HIV) (Srikumar et al., 2007) Furthermore, the ethanolic and aqueous extracts have shown antibacterial activity against the antibiotic resistant strains of bacteria and the aqueous extracts were more effective than ethanolic extracts. (Biradar et al., 2008)

2.1.6 Antihyperglycemic Effects

Today, diabetes affects about 5 percent of the global population and is the leading endocrine disease in the world. Animal studies show that the delivery of Triphala's equivalent concentration and the individual components decreased serum glucose concentrations in both usual and diabetic rats nduced by alloxane. (Sabu & Kuttan, 2007)

2.1.7 Antipyretic and Analgesic Activities

In a study the yeast-induced pyrexia in mice had decreased after administration of triphala, similar to indomethacin, which is the positive control. Triphala has also shown analgesic effect on mice which had acetic acid induced writhing and hot plate assay. (Sabina & Rasool, 2007)

2.1.8 Antioxidant Effects

The eukaryotic cells are loaded with natural antioxidant molecules like glutathione(GSH), vitamin E, vitamin A, carotenoids, vitamin C, etc. and antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase(GPx) to protect the cell from free radical-induced harm. Animal studies have shown that the administration of Triphala has raised SOD, GPx and CAT activities and increased GSH levels, that caused significant decline in the gastric tumors in mice.(Deep et al., 2005) Studies also show that Triphala reduced myeloperoxidase(MPO) and xanthine oxidase(XO) amounts in the intestinal mucosa

of methotrexate-treated rats with a simultaneous increase in the levels of GSH (Nariya et al., 2009)

Chapter 3

Methodology

3.1 Collection and Authentication

After investigation and having reviewed many literatures, it was strongly observed that many individual and combined studies have been conducted on these three fruits. Studies have shown that methanol has exposed the highest activity for antioxidants and against cancer cell line which is why methanol was chosen as the solvent. Fortunately, antioxidant activity of methanol extract in different ratios and cytotoxic activity against glioma (N4X4) cell line in 1:1:1 (A:B:H) and 2:1:1 (A:B:H) ratio of mixture was not used. The plant powders were obtained from commercially available packets.

3.2 Extraction Procedure

3.2.1 Extraction

The maceration process was used for extraction of plant materials and methanol was used as the organic solvent. 600 gm of the coarse powder of Amlaki, Haritaki and Bibhitaki was taken separately in three fresh, round bottomed flask (5 liters) and soaked in 1.2 liter of methanol each. With occasional agitation, the three materials in the flasks were soaked in methanol at normal room temperature (22-25°C) for a period of 7 days.

3.2.2 Filtration

The contents of the beaker were first decanted, after seven days of maceration, while being filtered using fresh cotton fabric, followed by cotton plug and Whitman filter (pore size: 110 mm).

3.2.3 Concentration and Drying

Using a rotary evaporator (Heidolph), the collected filtrate was concentrated at 100 rpm at 30°C until concentrated methanol extract was produced for each of the three extracts. The mixture was then transferred to a petri dish and placed under Laminar Air Flow (LAF) to evaporate the extract solvent, leaving the extract dry and semi-solid. LAF is also a form of prevention for microbial growth on the extract while it is drying. The petri dishes were covered in aluminum foil and labeled after successful drying and kept in a cool location.

3.3 *In vitro* Antioxidant Assay

There are various *in vitro* methods for estimation of antioxidant activities of plant extracts. DPPH free radical scavenging assay, total phenolic content (TPC) and total antioxidant capacity (TAC) methods were selected among the other methods to estimate the antioxidant activity in methanol extract of Triphala.

3.3.1 DPPH (1, 1-diphenyl-2-picryl hydrazyl) Free Radical Scavenging Assay

The DPPH free radical scavenging assay of methanol extract of Triphala was done using the method described by (Lalhmingmawii & Jagetia, 2018)

3.3.1.1 Reagents and Chemicals

The reagents and chemicals for the determination of DPPH scavenging activity are listed below in Table 5.

Table 5: Reagents and chemicals required to determine DPPH scavenging activity

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

3.3.1.2 Sample and Standard Solution Preparation

120 mg of extract was measured and dissolved in 10 mL of ethanol to produce a concentration of 12 mg/ mL. This became the stock solution and further concentrations were prepared by dilution of the sample stock solution to derive 8 concentrations: 1200, 800, 400, 200, 100, 50, and 25 µg/ mL. The standard used was L-ascorbic acid and it was prepared similarly to get 8 eight diluted concentrations, ranging from 1200-12.5 µg/ mL.

3.3.1.3 DPPH solution preparation

4 mg of DPPH was weighed and dissolved in 100 mL methanol to get 0.004% w/v of DPPH solution. This was then stored in the refrigerator at -4°C till before use.

3.3.1.4 Experimental Procedure

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

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

c. Control was prepared by adding 1 mL methanol and 2 mL DPPH.

d. 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 at 517 nm against blank (methanol) using a spectrophotometer (U- 2910 UV-Vis Spectrophotometer).

e. 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 = absorbance of the control
A_1 = absorbance of the sample/standard

f. Finally, the % Scavenging activity was plotted against concentration from which IC_{50} value was calculated.

g. 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 respective concentrations.

3.3.2 Determination of Total Phenolic Content (TPC)

The TPC of the plant extract of mixture was determined by the modified Folin-Ciocalteu method (Chandra et al., 2014).

3.3.2.1 Reagents and Chemicals

The reagents and chemicals for the determination of phenolic content are listed in Table 6.

Table 6: Reagents and chemicals required to determine flavonoid 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
Methanol	Active Fine Chemicals Ltd., Bangladesh

3.3.2.2 Reagent Preparation

100 mL of 10 % Folin-Ciocalteu reagent (FCR) solution was prepared by taking 10 mL of FCR in a volumetric flask and diluting it with distilled water. 100 mL of 7.5 % (w/v) Sodium carbonate was prepared by measuring out 7.5 g of sodium carbonate in a 100 mL volumetric flask and then diluting it with distilled water.

3.3.2.3 Sample and Standard Preparation

120 mg of extract was measured and dissolved in 10 mL of ethanol to produce a concentration of 12 mg/ mL. This became the stock solution and further concentrations were prepared by dilution of the sample stock solution to get four diluted concentrations: 1200, 800, 400 and 200 µg/ mL. Gallic acid was used as the standard and was prepared in the same manner as the extract.

3.3.2.4 Preparation of Blank

The blank solution contained 5 mL FCR solution and 4 mL sodium carbonate and 1 mL of ethanol was used to make the volume up to 10 mL.

3.3.2.5 Experimental Procedure

a. 0.5 mL of each of the fractions of sample and standard were taken in test tubes.
b. To each of the test tubes, 2.5 mL of FCR was added.
c. 2 mL of sodium carbonate solution was added.
d. Each of the mixtures was vortexed for 15 s and then allowed to stand for 30 min at 40°C in a water bath.
e. Finally, the absorbance of standard and sample solutions was measured against blank at 765 nm 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 equivalents (QE)

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)

3.3.3 Determination of Total Antioxidant Capacity (TAC)

The total Antioxidant capacity of plant extract of *Triphala* was determined by using the following method described by (Pisoschi & Negulescu, 2012).

3.3.3.1 Reagents and Chemicals

The reagents and chemicals for the determination of total antioxidant capacity are listed below in Table 7.

Table 7: Reagents and chemicals required to determine total antioxidant activity

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

3.3.3.2 Reagent Preparation

50 mL of 0.6 M Sulfuric acid was prepared by measuring 2.64 mL of 98 % concentrated sulfuric acid in a 50 mL volumetric flask and diluting it with distilled water. 50 mL of ammonium molybdate solution was prepared by measuring 2.247 g of ammonium molybdate in a 50 mL volumetric flask and diluting it with distilled water. 50 mL of 0.028 M trisodium phosphate solution was prepared by measuring 0.532 g of trisodium phosphate in a 50 mL volumetric flask and diluting it with distilled water.

3.3.3.3 Sample and Standard Preparation

120 mg of extract was measured and dissolved in 10 mL of ethanol to produce a concentration of 12 mg/ mL. This became the stock solution and further concentrations were prepared by dilution of the sample stock solution to get four diluted concentrations: 1200, 800, 400 and 200 µg/ 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 200 µg/ mL.

3.3.3.4 Preparation of the Blank

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

3.3.3.5 Experimental Procedure

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

b. 3 mL of Reagent solution (0.6 M sulfuric acid, 0.028 M sodium phosphate and 0.004 M 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 90 min.

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

e. The total antioxidant capacity, A, for each of the fractions were expressed as:

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

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 (mL), m = Weight of the sample (g)

3.4 Cytotoxicity Study by MTT Assay

3.4.1 N4X4 Cell Line

N4X4 cell line is a glioma NP-2 cell line with stable expression of CXCR4 produced in the M_{10} medium (MMM; Sigma, St. Louis, MO, USA) with a 10 % FBS supplement, 100 units/ mL penicillin and 0.1 mg/ mL streptomycin).

3.4.2 Solution Preparation

3.4.2.1 1% Penicillin–streptomycin Solution

Penicillin-streptomycin solution usually known as pen-strep is used in MTT assay to control the bacterial contamination and maintain the sterile condition throughout the process. The solution contains a mixture of 10000 units of penicillin per mL and 10 mg of streptomycin per mL. In case of N4X4 cell line, RPMI containing 1% penicillin-streptomycin is used where RPMI 1, also known as the RPMI medium, is a cell culture growth medium. In case of N4X4 cell line, DMEM containing 1% penicillin-streptomycin is used where, DMEM (Dulbecco's Modified Eagle Medium) is the most suitable medium for the culture of cells and tissues for many adherent cell phenotypes among defined media. The modification of Dulbecco is an enhanced additional formulation that boosts up to four-fold the selection of amino acid and
vitamin content of the original medium of Eagle.

3.4.2.2 10 % Fetal Bovine Serum

50 mL of Fetal Bovine Serum was added to the 500 mL of DMEM to prepare 10 % FBS. FBS is high in albumin that provides nutrient and adequate environment to grow the cells more quickly.

3.4.2.3 2% DMSO Solution

2 % DMSO solution was prepared by adding 600 µl in 29.4 mL of distilled water for negative control.

3.4.2.4 Trypsin

0.25 % trypsin was used in the medium.

3.4.2.5 Used Consumables

96 well plate, 15 mL tubes, Tips, Gloves, PTFE Syringe Filter, Culture flask, Cell culture media, 1% Penicillin-Streptomycin, Gentamycin, Serological pipette, Trypsin etc.

3.4.2.6 Used Instruments

Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA), CO2 incubator (Nuaire, USA), trinocular Microscope with Camera (Olympus, Japan), hemocytometer.

3.4.2.7 Celltiter 96 Assay Kit

Celltiter 96 assay kit is an accumulation of qualified reagents that is a fast and advantageous system for the determination of the amount of proliferation and cytotoxicity (Ifere et al., 2010). Under a formazan product, it shows the view of change of a cell division with tetrazolium salt, 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.

3.4.3 Preparation of the Different Concentrations of Plant Extract

The assay was conducted by using 5 concentration 25 mg/ mL, 2.5 mg/ mL, 0.25 mg/ mL and 0.025 mg/ mL and 0.0025 mg/ mL of Triphala extract in the ratio 1:1:1 (A:B:H) and 2:1:1 (A:B:H), where Amlaki was used in twice the amount. 25 mg/ mL concentration was made by placing in 25 mg of extract of each (total 75 mg) in 3 mL DMSO and it was the store solution. 2.5 mg/ mL concentration was completed by diluting

25 mg/ mL solution 10 times by DMSO. 30 µL of sample 1 is put in to the 270 µL of DMSO to make 2.5 mg/ mL concentration. 0.25 mg/ mL and 0.025 mg/ mL concentrations were completed by sequential dilution with. Later, the samples were sieved using a syringe filter of 0.45 µm pore size.

3.4.4 Cell Culture

3.4.4.1 Preparation of Assay Plates

N4X4 cell lines were maintained in RPMI and DMEM (Dulbecco's Modified Eagle's Medium) in addition with 1 % penicillin-streptomycin respectively, 0.2 % Gentamicin, 10 % fetal bovine serum.

3.4.4.2 Thawing of Cells

N4X4 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, the cells were suspended in the medium gently and transferred to the culture vessels. 90 µL of DMSO to make 2.5 mg/ mL concentration. 0.25 mg/ mL and 0.025 mg/ mL concentrations were completed by sequential dilution with. Later, the samples were sieved.

3.4.4.3 Cell Passage

To get a fresh cell suspension cell passaging was done by transferring the cells into a new medium. Thus, cultured media was washed by FBS and followed by addition of 800 µl 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 % ofcells 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.

3.4.4.4 Harvesting of Cells

The cells were harvested using trypin in log phase growth.

3.4.4.5 Counting of Cells

Hemocytometer was used for cell counting. The hemocytometer was prepared by cleaning and polishing the mirror like surface deliberately with ethanol and lens paper. The coverslip was put in the counting surface and then, using a pasteur pipet, the fresh cell suspension was introduced into the hemocytometer. Enough suspension was introduced for the surface to be merely overflowed. Afterward the counting chamber was set in the microscope stage then the counting grid was focused. In a standard hemocytometer (figure 7) with rulings 1 entire grid can be observed at 40X magnification. The cells present in the 4 large squares were counted afterwards. Either upper and left sides touching cells or lower or right sides touching cells were counted.

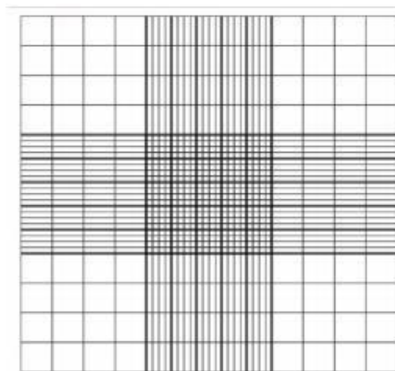

Figure 7: Hemocytometer

3.4.5 Procedure

Cytotoxic effect was performed in the Centre for Advanced Research in the Sciences (CARS) using their services. The celltiter 96 non- radioactive cell proliferation assay kit was used to perform the MTT colorimetric assay (Promega, USA). Cells were seeded onto 96 well plates and incubated at 37 °C and 5% carbon dioxide atmosphere. After 24 hours of incubation, 10μL of sample was added into each well. Then, 2 days of again incubation took place. After 2 days of incubation, cytotoxicity was examined using the assay kit and the absorbance was measured at 570 nm using a 96 Well Plate Reader. Same process was followed for the negative control and the 5 concentrations. Negative control contained medium with 2% DMSO solution and blank contained only the medium. Duplicate wells were used for each sample and finally the 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 4

Results

4.1 Determination of Percentage Yield of the Plant Extract *Triphala*

4.1.1 Total Weight of Powdered Plant Material

Table 8 represents the initial weight of powdered fruits used, the final extract and the % yield.

Table 8: % yield of Amlaki, Bibhttaki and Haritaki extracts

Sample	Initial weight of powder	Final weight of Extract	% Yield
Amlaki	600 g	88.23 g	14.71 %
Bibhitaki	600 g	55.33 g	9.22 %
Harikati	600 g	97.97 g	16.33 %

Interpretation: The % yield for Haritaki was the most compared to Amlaki and Bibhitaki. This is because more compound from Haritaki dissolved in the solvent used, which is methanol.

4.1.2 Calculation of Percentage Yield of the Extract

$$\%\ \text{yield} = \frac{\text{W1}}{\text{W2}} \times 100$$

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

W_2 = total weight of powder taken for extraction in gram

Percentage Yield of Bibhitaki

Percentage yield =

$$\frac{55.328}{600} \times 100$$

= 9.22 %

Percentage Yield of Amlaki

Percentage yield =

$$\frac{88.23}{600} \times 100$$

= 14.71 %

Percentage Yield of Haritaki

Percentage yield =

$$\frac{97.965}{600} \times 100$$

= 16.33 %

Interpretation: The percentage yield of leaf extract was found to be 9.22%, 14.71% and 16.33% from 600g of Bibhitaki, Amlaki and Haritaki respectively after the maceration process.

4.2 DPPH (1, 1-diphenyl-2-picryl hydroxyl) Free Radical Scavenging Assay

4.2.1 Table of DPPH free Radical Scavenging Activity of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala

The concentration and absorbance values of sample and standard (Ascorbic Acid) are given below in table 9:

Table 9: Table of DPPH Free Radical Scavenging Assay at 517 nm (Absorbance vs. concentration) of ascorbic acid, Bibhitaki, Amlaki and Haritaki

Concentration (μg/ mL)	Absorbance of Ascorbic Acid (Standard)	Absorbance of Bibhitaki	Absorbance of Amlaki	Absorbance of Haritaki
3.125	0.608	0.647	0.598	0.611
6.25	0.457	0.447	0.438	0.583
12.5	0.312	0.275	0.256	0.283
25	0.129	0.134	0.088	0.178
50	0.053	0.052	0.043	0.048
100	0.039	0.042	0.029	0.039
200	0.029	0.033	0.028	0.038
400	0.029	0.031	0.026	0.033
800	0.024	0.031	0.025	0.031
1200	0.023	0.032	0.024	0.030

The Table 9 above shows the absorbance values of Bibhitaki, Amlaki, Haritaki and standard corresponding to their respective concentrations at 517nm. The standard deviations are also stated. The concentrations are ranging from 3.125μg/ mL to 1200μg/ mL.

Table 10: Table of DPPH Free Radical Scavenging Assay at 517 nm (Absorbance vs. concentration) of the different ratios of Triphala

Concentration (µg/ mL)	Absorbance of 1:1:1 (A:B:H)	Absorbance of 2:1:1 (A:B:H)	Absorbance of 1:2:1 (A:B:H)	Absorbance of 1:1:2 (A:B:H)
3.125	0.564	0.548	0.626	0.694
6.25	0.426	0.370	0.396	0.354
12.5	0.326	0.262	0.173	0.227
25	0.127	0.104	0.109	0.147
50	0.058	0.053	0.030	0.037
100	0.026	0.018	0.027	0.035

The Table 10 above shows the absorbance values of the different Triphala ratios corresponding to their respective concentrations at 517nm. The standard deviations are also stated. The concentrations are ranging from 3.125 µg/ mL to 100µg/ mL.

4.2.2 Graph of Absorbance vs. Concentration of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala in DPPH free radical scavenging assay

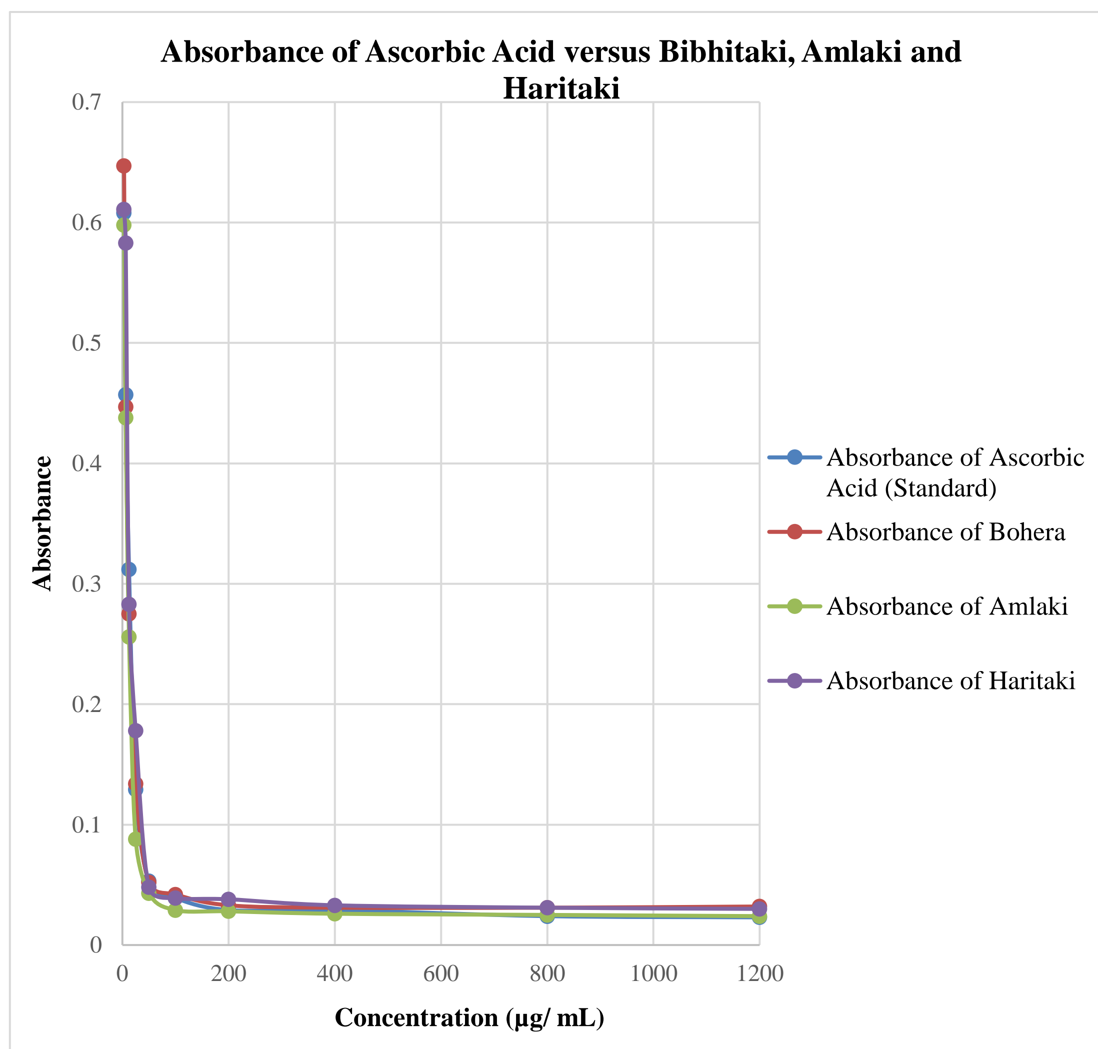

Figure 8: Graph of Absorbance vs. Concentration (µg/ mL) for Ascorbic Acid, Bibhitaki, Amlaki and Haritaki in DPPH free radical scavenging assay

Interpretation: From the Table 9 and Figure 8 above, it was observed that with an increase in concentration of extracts from 3.125– 1200µg/ mL, the absorbance decreased gradually. However, after 100µg/ mL the absorbance decrease was not as drastic as before and only small differences could be seen. Moreover, the graphs of all the extracts have similar slope for Absorbance vs. Concentration (µg/ mL).

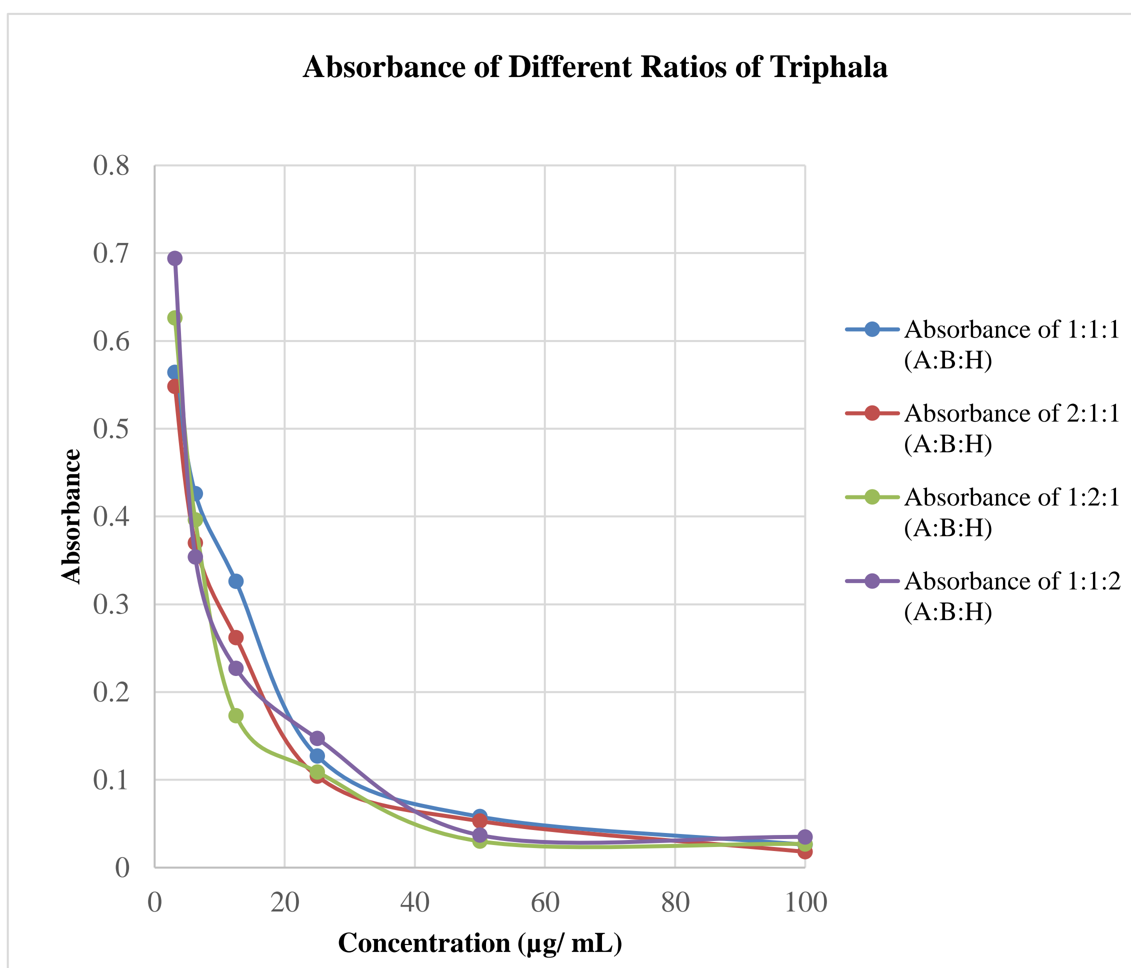

Figure 9: Graph of Absorbance vs. Concentration (µg/ mL) for different ratios of Triphala in DPPH free radical scavenging assay

Interpretation: In Figure 9 it was observed that with an increase in concentration from 3.125– 100µg/ mL, the absorbance decreased gradually for all the ratios. The absorbance of 1:2:1 (A:B:H) has the steepest slope shown in green compared to the standard shown in blue.

4.2.3 Table of Percentage of Inhibition of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and different ratios of Triphala

Table 11: Table of % Inhibition of DPPH Free Radical by Ascorbic Acid, Bibhitaki, Amlaki and Haritaki

Concentration (µg/ mL)	% inhibition of Ascorbic Acid (Standard)	% inhibition of Bibhitaki	% inhibition of Amlaki	% inhibition of Haritaki
3.125	20.209	15.092	21.522	19.816
6.25	40.0263	41.339	42.519	23.491
12.5	59.055	63.911	66.404	62.861
25	83.071	82.415	88.451	76.640
50	93.045	93.176	94.357	93.701
100	94.882	94.488	96.194	94.882
200	96.194	95.669	96.325	95.013
400	96.194	95.722	96.588	95.669
800	96.850	95.831	96.719	95.932
1200	96.982	95.841	96.850	96.063

Interpretation: In Table 11 it was observed that for concentrations 3.125 to 100 µg/ mL, the % inhibition of free radical scavengers gradually increased. However, from 200 to 1200 µg/ mL, the values for both the standard and samples remained nearly constant. 95.801 %, 96.850% and 96.063% of inhibition of free radical was found for Bibhitaki, Amlaki and Haritaki respectively, at 1200 µg/ mL.

Table 12: Table of % Inhibition of DPPH Free Radical by different ratios of Triphala

Concentration (µg/ mL)	% inhibition of 1:1:1 (A:B:H)	% inhibition of 2:1:1 (A:B:H)	% inhibition of 1:2:1 (A:B:H)	% inhibition of 1:1:2 (A:B:H)
3.125	25.984	28.083	17.847	8.923
6.25	44.094	51.443	48.031	53.543
12.5	57.218	65.616	77.296	70.209
25	83.333	86.351	85.695	80.708
50	92.388	93.044	96.062	95.144
100	96.587	97.637	96.456	95.406

Interpretation: In Table 12 it was observed that for concentrations 3.125 µg/ mL to 100 µg/ mL the % inhibition of free radical scavengers by ascorbic acid and the extract ratios gradually increased. The highest free radical scavenging activity for the sample ratios were found to be at 100µg/ mL and the percentage inhibition was 96.587%, 97.637%, 96.456% and 95.406% for the Triphala ratios 1:1:1(A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H) and 1:1:2 (A:B:H) respectively.

4.2.4 Graph of % Inhibition of DPPH Free Radical by Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala

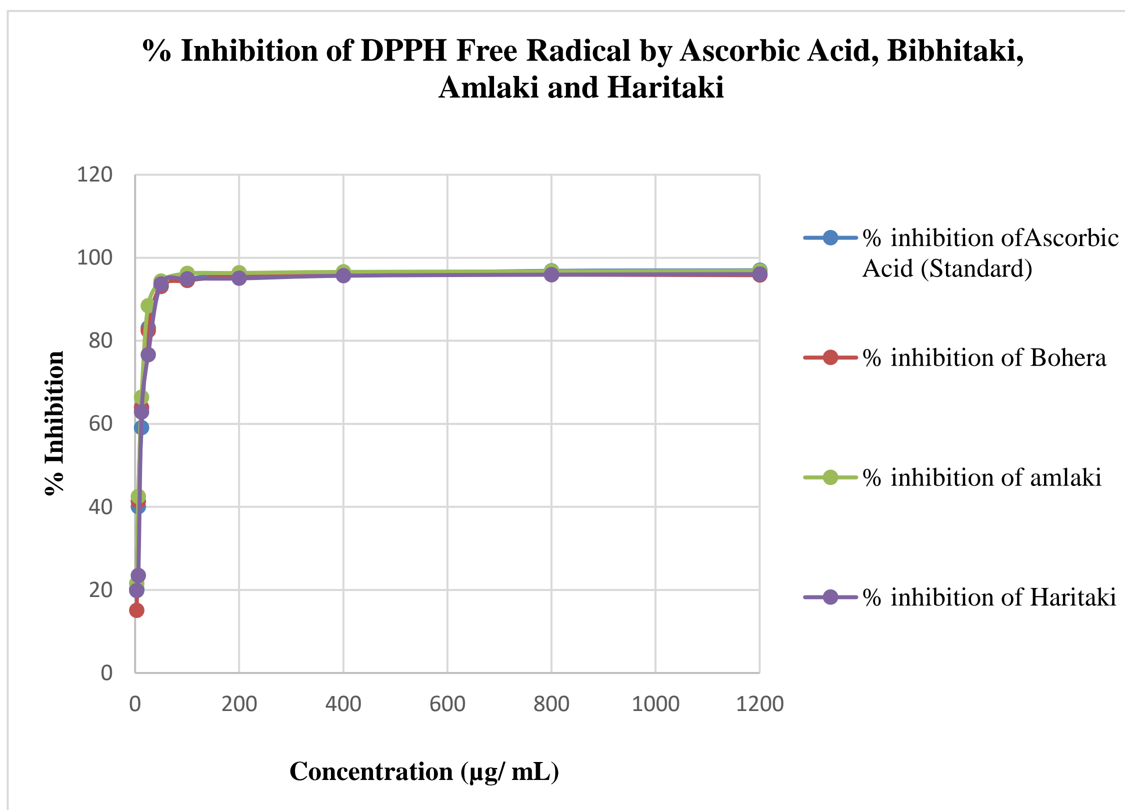

Figure 10: Graph of % Inhibition of DPPH Free Radical by Ascorbic Acid, Bibhitaki, Amlaki and Haritaki

Interpretation: In Figure 10 we can see the % inhibitory activity has increased from concentration ranging from 3.125 to 100µg/ 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. All the extracts have similar slope of % inhibition.

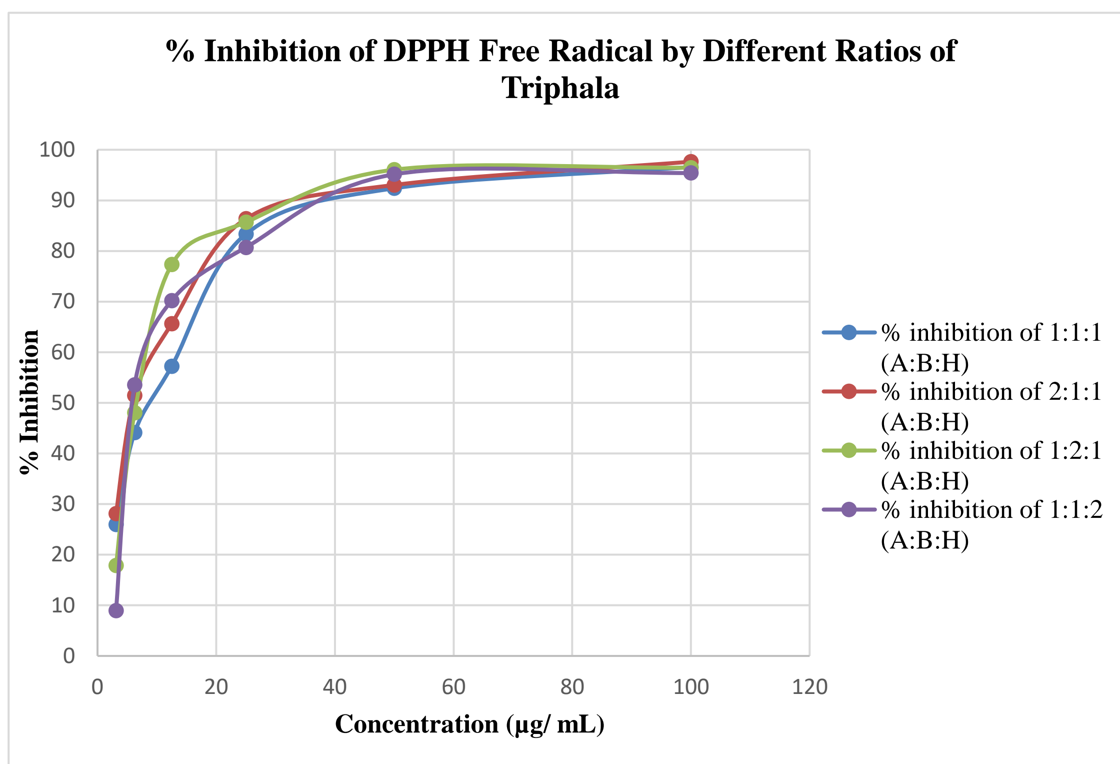

Figure 11: Graph of % Inhibition of DPPH Free Radical by different ratios of Triphala

Interpretation: In Figure 11 we can see the % inhibitory activity has increased from concentration ranging from 3.125 to 100µg/ mL. In conclusions, it can be said that with increasing concentrations, the percentage of inhibition did increase proportionally. The % inhibition of 1:2:1 (A:B:H) shown in green has the steepest slope until it starts to decline.

4.2.5 Determination of the IC_{50} of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala

Table 13: IC_{50} values of Ascorbic Acid, Bibhitaki, Amlaki, Haritaki and Different Ratios of Triphala

Samples and Standard	IC_{50} value in μg/ mL
Ascorbic acid (Standard)	12.96
Haritaki	15.93
Bibhitaki	12.99
Amlaki	10.287
1:1:1 (A:B:H) ratio	11.28
2:1:1 (A:B:H) ratio	7.01
1:2:1 (A:B:H) ratio	8.33
1:1:2 (A:B:H) ratio	11.03

Interpretation: In Table 13 it can be observed that 15.93 μg/ mL, 12.99 μg/ mL and 10.287 μg/ mL of Haritaki, Bibhitaki and Amlaki was found to inhibit 50 % of DPPH free radical scavengers, which is the IC_{50} value. As the IC_{50} value for Ascorbic Acid was 12.96 μg/ mL, Amlaki showed more inhibition at a lower concentration. The IC_{50} value for the Triphala ratios 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H) and 1:1:2 (A:B:H) were 11.28 μg/ mL, 7.01 μg/ mL, 8.33 μg/ mL and 11.03 μg/ mL respectively and it was observed that all the values were lower than that of the standard and the individual components of Triphala, with 2:1:1 (A:B:H) being the lowest and most inhibitory. This suggested synergistic effect when the three extracts are mixed.

4.3 Total Phenolic Content (TPC) Determination

4.3.1 Calibration Curve of Gallic Acid at 765 nm

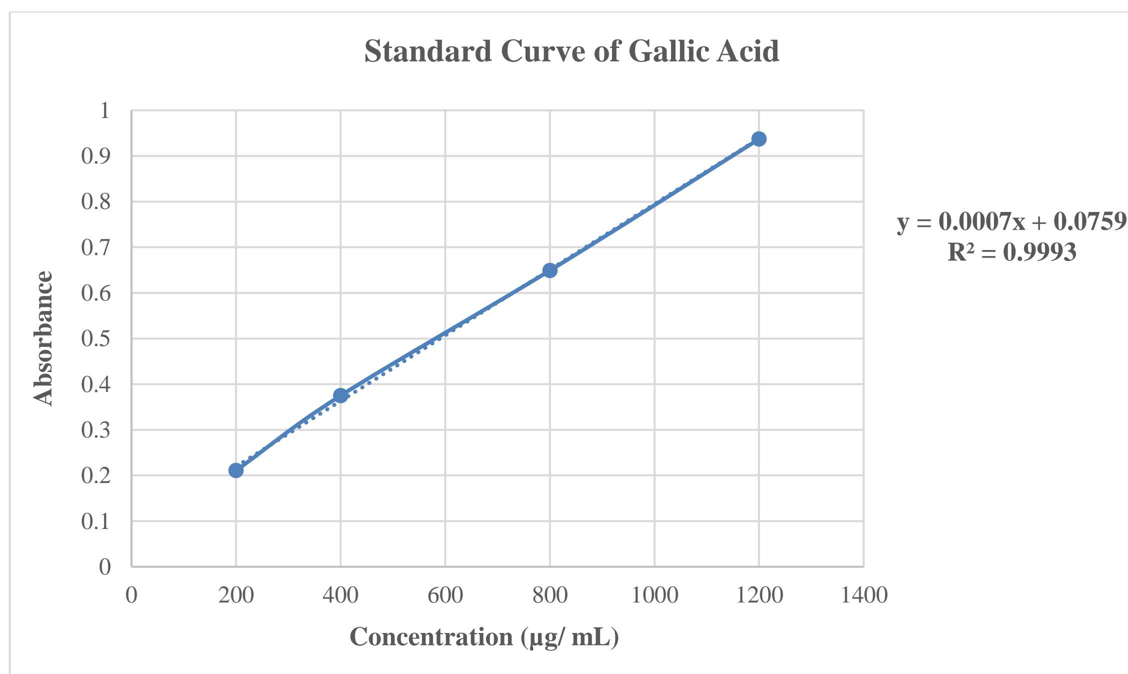

Figure 12: Standard curve of standard gallic acid at 765 nm to determine the TPC in sample

4.3.2 Table of total phenolic content in Triphala

Table 13: Total phenolic content (TPC) of Bibhitaki is marked as gallic acid equivalent (GAE)

Concentration of Sample (µg/ mL)	Total Phenolic Content at 765 nm of Bibhitaki	Total Phenolic Content at 765 nm of Amlaki	Total Phenolic Content at 765 nm of Haritaki
200	190.66	178.9	37.51
400	210.37	243.94	220.66
800	349.66	348.23	254.66
1200	368.94	395.09	260.65

Interpretation: It is observed from Table 13 and Figure 12 that, concentration of samples has proportional relationship with the TPC. When the concentration (200 µg/ mL to 1200 µg/ mL) of methanol extract of samples increased, total phenolic content also increased from 190.66 mg to 368.94 mg, 178 mg to 395.09 mg and 37.51 mg to 260.65 mg of gallic acid equivalent per gram of dried extract for Bibhitaki, Amlaki and Haritaki respectively. Moreover, it indicates that with the increase of total phenolic content, its antioxidant activity also increases.

4.4 Total Antioxidant Capacity Determination

4.4.1 Calibration curve of Ascorbic Acid (Standard) at 695 nm

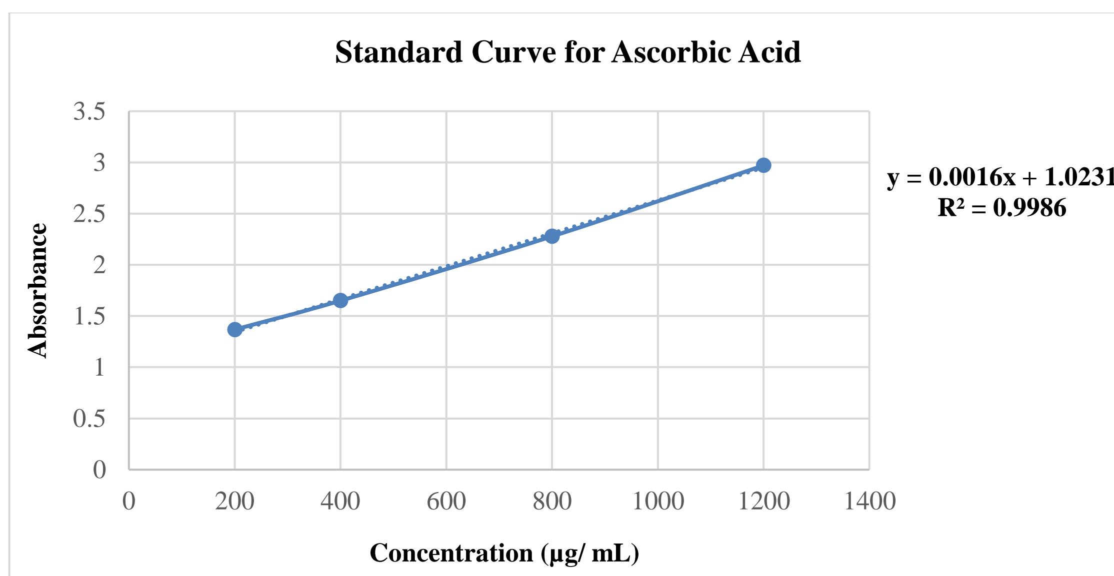

Figure 13: Standard curve of ascorbic acid at 695 nm for determining TAC value in methanol extract of Triphala

4.4.2 Table of Total Antioxidant Capacity (TAC) in Triphala

Table 14: Total antioxidant content (TAC) of Triphala is marked as Ascorbic Acid equivalent (AAE)

Concentration of Sample (μg/ mL)	Total Antioxidant Capacity (TAC) at 695 nm of Bibhitaki	Total Antioxidant Capacity (TAC) at 695 nm of Amlaki	Total Antioxidant Capacity (TAC) at 695 nm of Haritaki
200	8.51	16.95	90.075
400	121.39	163.2	154.76
800	197.14	235.95	417
1200	245.32	485.5	559.76

Interpretation: It is observed from the table 14 and figure 13, concentration of *Triphala* has proportional relationship with the TAC. When the concentration (200 μg/ mL to 1200 μg/ mL) of methanol crude extract of Triphala increased, total antioxidant capacity also increased from 8.51 to 245.32 mg, 16.95 to 485.5 mg and 90.075 to 559.76 mg of ascorbic acid equivalent per gram of dried extract for Bibhitaki, Amlaki and Haritaki respectively. Moreover, it indicates that with the increase of total flavonoid content, its antioxidant activity also increases.

4.5 In vitro Cytotoxicity Test of Triphala by MTT Assay on N4X4 (Human Glioma) Cell Line

The cytotoxicity activity of methanol extract of Triphala in 1:1:1 (A:B:H) and 2:1:1 (A:B:H) ratio was accomplished by MTT assay on N4X4 cell line. Different concentrations (25 mg/ mL, 2.5 mg/ mL, 0.25 mg/ mL and 0.025 mg/ mL) of the methanol extract of sample were used to investigate the cytotoxic activity. 2 % DMSO in DMEM medium was used as a negative control. Absorbance along with their average

was observed for each of the concentrations. Survival of cells and % of growth inhibition of N4X4 cell line in different concentrations along with their IC_{50} value are given in the table 15. Images were also captured which are attached in figure 14.

4.5.1 Cytotoxicity Test on N4X4 Cell Line using 1:1:1 (A:B:H) ratio

Table 15: Survival and inhibition of N4X4 cell line in different concentrations along with their IC_{50} value and average absorbance using the 1:1:1 (A:B:H) ratio

Sample Concentration (mg/ mL) 1:1:1 (A:B:H) ratio	Average Absorbance	Survival of N4X4 Cell (%)	N4X4 Cell Growth Inhibitions (%)	IC_{50} Value (mg/ mL)
2% DMSO (cell + solvent)	3.754	100	0	13.43
25	0.418	11.12	88.88	
2.5	3.308	88.11	11.89	
0.25	3.551	94.57	5.43	
0.025	3.589	95.61	4.39	
0.0025	3.606	96.04	3.96	

Interpretation: Table 15 shows the percentage of the survival of the N4X4 cell that was observed through the trinocular microscope. When the concentration of sample extract was 25 mg/ mL, highest cell death was detected (88.88 % cell death where survival of N4X4 cells were 11.12 %) after 48 hours of incubation. Extracts exhibited weak cytotoxic activity at the other four concentrations of 2.5 mg/ mL, 0.25 mg/ mL, 0.025 mg/ mL and 0.0025 mg/ mL, as the percentage of inhibition was 11.89%, 5.43%, 4.39% and 3.96 % respectively.

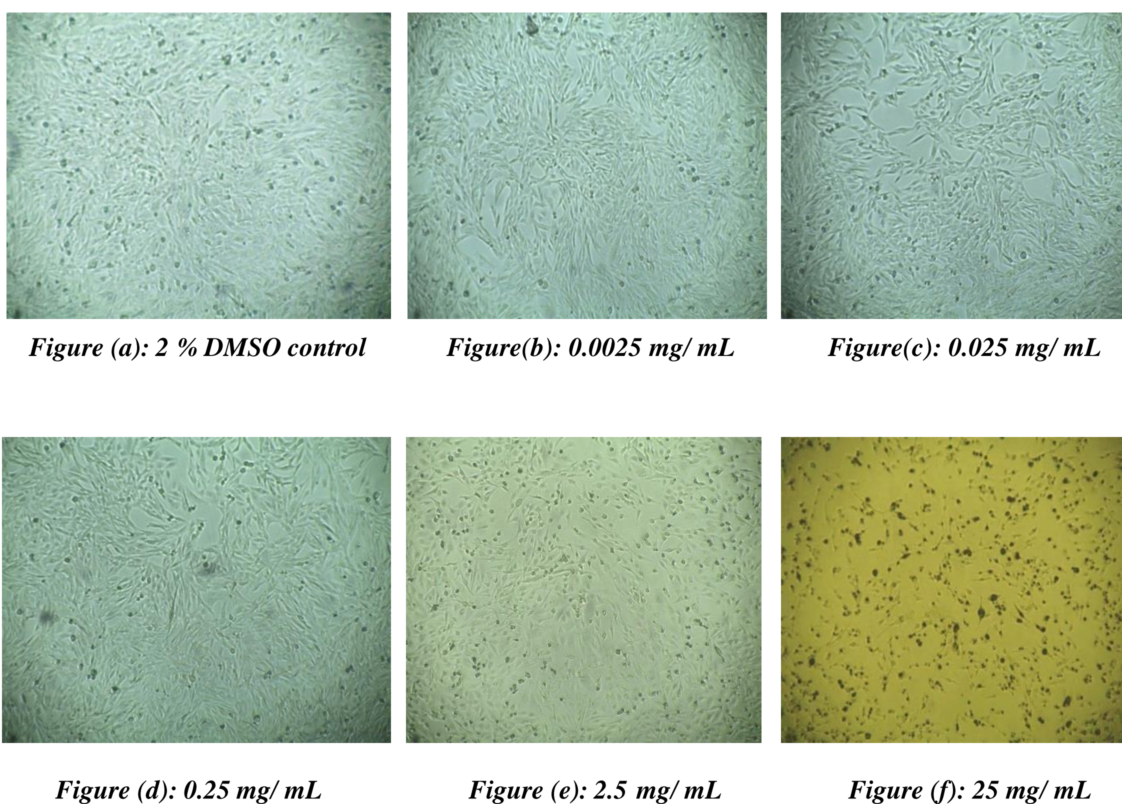

Figure (a): 2 % DMSO control ***Figure(b): 0.0025 mg/ mL*** ***Figure(c): 0.025 mg/ mL***

Figure (d): 0.25 mg/ mL ***Figure (e): 2.5 mg/ mL*** ***Figure (f): 25 mg/ mL***

Figure 14: Cell viability of methanol extract of Triphala, 1:1:1(A:B:H) ratio, at different extract concentrations of 0.0025 mg/ mL, 0.025mg/ mL, 0.25 mg/ mL, 2.5 mg/ mL, 25 mg/ mL and 2% DMSO as control respectively after incubating 48 hours in N4X4 cell line

Interpretation: Figure 14 illustrates the cell viability of N4X4 cells in different concentrations of the plant extracts as well as in the control (2 % DMSO). Figure (a) represents the control where 2 % DMSO was added and no plant extract was included. In the figure (b), cell viability decreased due to adding leaf extract of 0.0025 mg/ mL concentration; only 3.96 % cell growth inhibition was perceived. Slightly less cell viability was observed while 0.025 mg/ mL and .25mg/ mL concentration of the extract was added in figure (c) and (d). In the next picture figure (e), visible cell growth inhibition was observed due to adding 2.5mg/ mL concentration where percentage of cell growth inhibition was 11.89%. Finally, in the last picture, highest cytotoxicity of plant extract was observed as it killed maximum number of cells and percentage of cell

growth inhibition was highest; approximately 88.88 %. It depicts that, plant extract of 25 mg/ mL concentration shows a remarkable cytotoxic potential with 88.88 % cell inhibition.

4.5.2 Cytotoxicity Test on N4X4 Cell Line using 2:1:1 ratio (A:B:H)

Table 16: Survival and inhibition of N4X4 cell line in different concentrations along with their IC_{50} value and average absorbance using 2:1:1 (A:B:H) ratio

Sample Concentration (mg/ mL) 2:1:1 (A:B:H) ratio	Average Absorbance	Survival of N4X4 Cell (%)	N4X4 Cell Growth Inhibition (%)	IC_{50} (mg/ mL)
2% DMSO (cell + solvent)	3.754	100	0.00	13.23
25	0.439	11.25	88.75	
2.5	0.364	85.48	14.52	
0.25	3.209	95.07	4.93	
0.025	3.644	97.06	2.94	
0.0025	3.753	99.98	0.02	

Interpretation: Table 16 shows the percentage of the survival of the N4X4 cell that was observed through the trinocular microscope. When the concentration of sample extract was higher (25 mg/ mL), highest cell death was detected of 88.75 %, where survival of N4X4 cells were 11.25 % after 48 hours of incubation. Extracts exhibited weak cytotoxic activity at the other four concentrations of 2.5 mg/ mL, 0.25 mg/ mL, 0.025 mg/ mL and 0.0025 mg/ mL, as the percentage of inhibition was 14.52%, 4.93%, 2.94% and 0.02 % respectively.

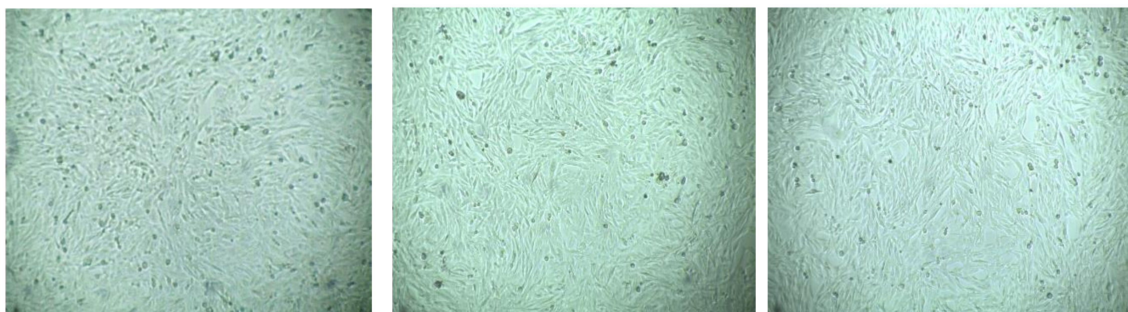

Figure (a): 2 % DMSO control ***Figure (b): 0.0025 mg/ mL*** ***Figure(c): 0.025 mg/ mL***

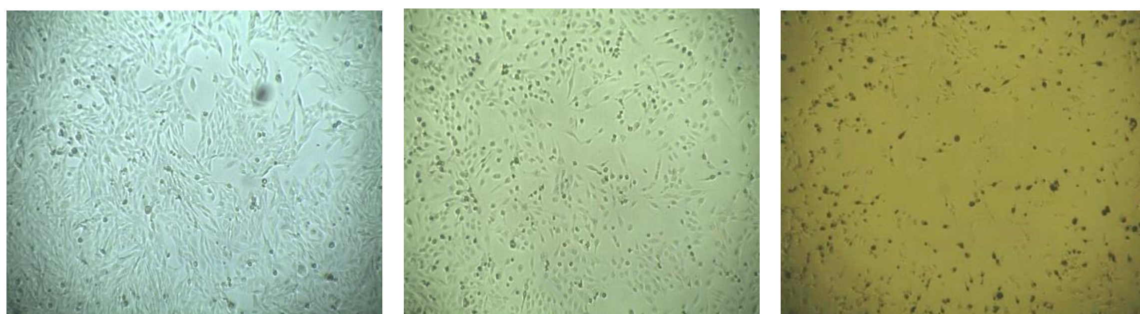

Figure (d): 0.25 mg/ mL ***Figure (e): 2.5 mg/ mL*** ***Figure (f): 25 mg/ mL***

Figure 15: Cell viability of methanol extract of Triphala, 2:1:1 (A:B:H) ratio, at different extract concentrations of 0.0025 mg/ mL, 0.025mg/ mL, 0.25 mg/ mL, 2.5 mg/ mL, 25 mg/ mL and 2% DMSO as control respectively after incubating 48 hours in N4X4 cell line

Interpretation: Figure 15 illustrates the cell viability of N4X4 cells in different concentrations of the plant extracts as well as in the control (2 % DMSO). Figure (a) represents the control where 2 % DMSO was added and no plant extract was included. In the figure (b), cell viability decreased due to adding leaf extract of 0.0025 mg/ mL concentration; only 0.02 % cell growth inhibition was perceived. Slightly less cell viability was observed while 0.025 mg/ mL and .25mg/ mL concentration of the extract was added in figure (c) and (d). In the next picture figure (e), visible cell growth inhibition was observed due to adding 2.5mg/ mL concentration where percentage of cell growth inhibition was 14.52%. Finally, in the last picture, highest cytotoxicity of plant extract was observed as it killed maximum number of cells and percentage of cell

growth inhibition was highest; approximately 88.75 %. It depicts that, plant extract of 25 mg/ mL concentration shows a remarkable cytotoxic potential with 88.75 % cell inhibition.

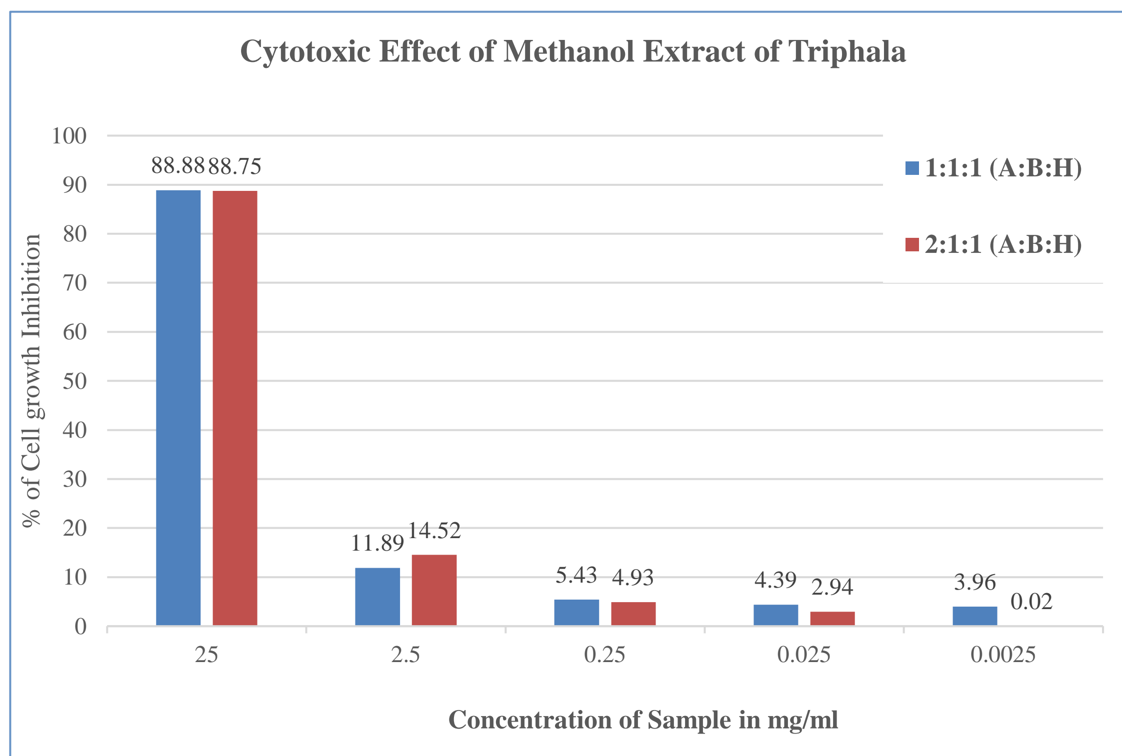

Figure 16: Cytotoxic effect of methanol crude extract on N4X4 cell line

Interpretation: The figure 16 complies the graphical representation of the percentage of cell growth inhibition vs. the respective concentration samples. From the bar chart, it was observed that the lowest percentage of cell inhibition was at 0.0025 mg/ mL concentration of plant extract. On the other hand, the highest percentage of cell inhibition was observed at 25 mg/ mL concentration of plant extract. The % inhibition of the two ratios of Triphala are almost the same and so change in concentration of Amlaki did not increase % inhibition of cell growth.

Chapter 5

Discussion

This study was conducted with Triphala, which is a mixture of Amlaki, Bibhitaki and Haritaki. 600 gm of individual fruit powder of Amlaki, Bibhitaki and Haritaki were soaked in methanol and the weight of extract after evaporation and drying was 88.23g, 55.33g and 97.97g, thus giving a percentage yield of 14.71%, 9.22% and 16.33% respectively. The higher percentage yield of Haritaki is because more compounds were able to dissolve in methanol. Moreover, methanol solvent was never used before to estimate the antioxidant activity by TAC and TPC and DPPH assay was not conducted on different ratios of Triphala to compare with the equivalent mixture. That is why it is one of the appropriate choices to determine the antioxidant potential of methanol extract. *In vitro* antioxidant tests such as DPPH free radical scavenging assay, Total Phenolic Content (TPC) and Total Antioxidant Capacity (TAC) were carried out to examine the antioxidant potential of methanol extract of Amlaki, Bibhitaki and Haritaki. Furthermore DPPH free radical scavenging assay was also carried out for different ratios of Triphala which were, 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H) and 1:1:2 (A:B:H). In DPPH test, the highest percentage of inhibition of free radical was found at the highest concentration of 1200 μg/ mL which was 95.801 %, 96.850% and 96.063% of inhibition of free radical was found for Bibhitaki, Amlaki and Haritaki. Furthermore, at 100 μg/ mL, which is the highest concentration, the percentage inhibition was 96.587%, 97.637%, 96.456% and 95.406% for the Triphala ratios 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H) and 1:1:2 (A:B:H) respectively. As the percentage inhibition of the ascorbic acid (standard) was 96.982%, only 2:1:1 (A:B:H) Triphala ratio showed more inhibition which was 97.637%. From the perspective of

IC_{50} value, 15.93 μg/ mL, 12.99 μg/ mL and 10.287 μg/ mL of Haritaki, Bibhitaki and Amlaki was found to inhibit 50 % of DPPH free radical scavengers. As the IC_{50} value for Ascorbic Acid was 12.96 μg/ mL, Amlaki showed more inhibition at a lower concentration. The IC_{50} value for the Triphala ratios 1:1:1(A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H) and 1:1:2 (A:B:H) were 11.28 μg/ mL, 7.01 μg/ mL, 8.33 μg/ mL and 11.03 μg/ mL respectively and it was observed that all the values were lower than ascorbic acid and the individual components of Triphala as well. The ratio 2:1:1 (A:B:H) gave the lowest value and was the most inhibitory. This suggested synergistic effect when the three extracts are mixed together.

In total phenolic test (TPC) and total antioxidant test, gallic acid and ascorbic acid was used as the standard. In case of total phenolic test, at concentration of 1200 μg/ mL, dried extract showed highest amount which was 368.94mg, 395.09mg and 260mg of gallic acid per gram for Bibhitaki, Amlaki and Haritaki respectively. Finally, for total antioxidant test, at the concentration of 1200 μg/ mL, dried extract showed highest amount which was 245.32mg, 485.5mg and 559.76mg of ascorbic acid per gram for Bibhitaki, Amlaki and Haritaki respectively. From the values obtained from the tests, we can conclude that Amlaki had the highest gallic acid per gram of 395.09mg and Haritaki had the highest ascorbic acid per gram of 559.76mg.

Additionally, cancer cell lines were selected for screening the cytotoxic activity before such as Michigan Cancer Foundation-7, breast cancer cells, prostate cancer cells, human pancreatic cancer cells, mouse breast cancer cells and mouse thymic lymphoma (Kaur et al., 2005; Sandhya et al., 2006; Sandhya & Misha, 2006; Kaur et al., 2005, Shi et al., 20008; Sandhya et al., 2006). Among all of the solvents used for screening, methanol extract showed highest percentage in case of cytotoxicity potentiality. However, N4X4 cell line related to brain cancer were not used previously in order to

check the cytotoxic activity of Triphala. Furthermore, the ratio 2:1:1 (A:B:H) was also used alongside the ratio 1:1:1 (A:B:H) to compare and see if using more Amlaki, which has the highest gallic acid among the three and is also more easily edible, would show more cytotoxicity. Gallic acid is one of the major components of Triphala and capable of inhibiting cancer cell proliferation suggesting the key factor responsible for antimutagenic and cytotoxic effects of Triphala (Kaur et al., 2005).

Afterwards, *in vitro* cytotoxicity test known as MTT assay was conducted on N4X4 cell line where cytotoxicity was examined after 48 hour of incubation and 2% DMSO was used as the negative control. C and percentage of cell growth inhibition was determined. and 25 mg/ mL concentration of extract provided the highest cytotoxic activity where percentage of cell death was 88.88 % for 1:1:1 (A:B:H) and 88.75% for 2:1:1 (A:B:H). Data interpretation also revealed that 50 % of cell death required 13.43 mg/ mL in case of 1:1:1 (A:B:H) and 13.23 mg/ mL for 2:1:1 (A:B:H) of extract concentration meaning the extract showed moderate cytotoxic activity in N4X4 cell line. The percentage of cell growth inhibition was similae for both the ratios and so, more Amlaki did not give better results as their cytotoxic activity was almost the same with a small difference of .20 mg.

Triphala (MET) exhibited dose dependent antiproliferative properties in cancer stem cells (HCCSC) and cancer cells (HCT116) and apoptotic properties in HCCSCs. Apoptosis induction by Triphala was via the mitochondrial apoptotic signaling characterized by elevated Bax/Bcl-2 ratio. These results indicate that Triphala (MET) may serve as an effective anticancer agent, mainly against colon cancer and has a potential to be taken simultaneously to conventional chemotherapy in the management of colon cancer (Vadde et al., 2015). The discrepancies however may be due to usage of different *in- vitro* methods, variation in concentration and other physiological factors

which lead to less activity against N4X4 cancer cell line. On a concluding note, methanol extract of Triphala confirms highest antioxidant activity and moderate cytotoxic activity. So, methanol extract can be used as a promising antioxidant agent, but it is not highly suggested as anticancer agent to treat brain tumour.

Chapter 6

Conclusion

In DPPH test, highest percentage of inhibition of free radical was found at the highest concentration which was 1200 µg/ mL. The IC_{50} values were 15.93 µg/ mL, 12.99 µg/ mL and 10.287 µg/ mL of Haritaki, Bibhitaki and Amlaki, for Ascorbic Acid it was 12.96 µg/ mL. The IC_{50} value for the Triphala ratios 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H) and 1:1:2 (A:B:H) were 11.28 µg/ mL, 7.01 µg/ mL, 8.33 µg/ mL and 11.03 µg/ mL respectively and it was observed that all the values were lower than that of the standard and the individual components of Triphala. The 2:1:1 (A:B:H) showed the lowest value among all the samples. This suggested synergistic effect when the three extracts are mixed together. Thus, this provides the evidence that small amount of concentrations of is required to inhibit the DPPH free radicals and shows strong antioxidant activity against free radicals. Total phenolic content and total antioxidant capacity were also found strong compared to the standard through the conducted experiments. Thus, Triphala showed high antioxidant activity in all of the testes. Additionally, N4X4 cell line was used to investigate the cytotoxic potential of Triphala and moderate cell toxicity was observed at 25 mg/ mL concentration of the two ratios. To conclude, methanol extract of Triphala signifies high antioxidant activity and moderate cytotoxic potential and can be used to treat oxidative degenerative diseases but cannot be used effectively for brain cancer until further refinement of useful

compounds from this extract can be done.

Future Direction

The result from this study established that methanol extract of Triphala implied very strong antioxidant activity compared to the standard ascorbic acid. On the other hand, Triphala possessed moderate cytotoxic activity.

- Other ratios of the Triphala extract can be used to find cytotoxicity
- Triphala extract can be used in different ratios to see it’s chemoprotective and radioprotective capacity
- Synergistic outcomes can be highlighted properly with the use of methanol extract of Triphala.
- Other plant parts such as root, stem can be used to investigate the cytotoxic and antioxidant potential using the same methanol solvent.
- High amount of antioxidant activity shows scope to further isolate and analyze new compounds.

References

Ahmed, Z. S. (2016). Investigation of in-vitro antioxidant potential in crotalaria verrucosa along with identification and quantification of its polyphenolic compounds. Retrieved from http://dspace.bracu.ac.bd/xmlui/handle/10361/7892

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharmaceutical Journal, 21(2), 143–152. https://doi.org/10.1016/J.JSPS.2012.05.002

Alphandéry, E. (2018). Glioblastoma Treatments: An Account of Recent Industrial Developments. Frontiers in Pharmacology, 9, 879. https://doi.org/10.3389/fphar.2018.00879

Aung TN, Zhipeng Q, Daniel Kortschak R, Adelson DL. Understanding the effectiveness of natural compound mixtures in cancer through their molecular mode of action. International Journal of Molecular Sciences. 2017;18(656):1-20

Amrithpal Singh Saroya. Herbalism phytochemistry and Ethanopharmacology, Science Publishers. 2011;357-361.

Amrithpal Singh. Medicinal Plants of the World, Published by Mohan Pri mLani for Oxford and IBH Co. Pvt,New Delhi.2006; 26.

Balunas, M. J., & Kinghorn, A. D. (2005). Drug discovery from medicinal plants. Life Sciences, 78(5), 431–441. https://doi.org/10.1016/j.lfs.2005.09.012

Biradar YS, Jagatap S, Khandelwal KR, Singhania SS. 7. Exploring of antimicrobial activity of Triphala mashi –an Ayurvedic Formulation. Evid Based Complement Alternat Med 2008;5:107-113.

Boer, E., et al. 1995. Terminalia L. In Lemmens, R.H.M.J., Soerianegara, I. & Wong,

W.C. (Eds.): Plant Resources of South-East Asia. No. 5(2): Timber tree: Minor commercial timber. Prosea Foundation, Bogor, Indonesia. pp. 475-478, 483. 21.

Calixto, J., Santos, A., Filho, V., & Yunes, R. (1998). A review of the plants of the genusPhyllanthus: Their chemistry, pharmacology, and therapeutic potential. Medicinal Research Reviews, 18(4), 225-258. doi: 10.1002/(sici)1098-1128(199807)18:4<225::aid-med2>3.0.co;2-x

Chandra, S., Khan, S., Avula, B., Lata, H., Yang, M. H., Elsohly, M. A., & Khan, I. A. (2014). Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: a comparative study. Evidence-Based Complementary and Alternative Medicine: ECAM, 2014, 253875. https://doi.org/10.1155/2014/253875

Chandra Gupta P, Biological and pharmacological properties of Terminalia chebula Retz. (Haritaki)- an overview,Int J Pharm Pharm Sci 2012; 4(3): 62-68.

Chattopadhyay RR, Bhattacharyya SK. Plant Review Terminalia chebula. Pharmacognos. Rev. 2007. 23:145-15

Chiang, L.-C., Cheng, H.-Y., Chen, C.-C., & Lin, C.-C. (2004). In vitro Anti-leukemic and Antiviral Activities of Traditionally Used Medicinal Plants in Taiwan. The American Journal of Chinese Medicine, 32(05), 695–704. https://doi.org/10.1142/S0192415X04002284

Deep G, Dhiman M, Rao AR, Kale RK. Chemopreventive 29. potential of Triphala (a composite Indian drug) on benzo(a) pyrene induced forestomach tumorigenesis in murine tumor model system. J Exp Clin Cancer Res 2005;24:555-563.

Deep G, Dhiman M, Rao AR, Kale RK. Chemopreventive 29. potential of Triphala (a composite Indian drug) on benzo(a) pyrene induced forestomach tumorigenesis in murine tumor model system. J Exp Clin Cancer Res 2005;24:555-563.

Gore, M., & Desai, N. S. (2014). Characterization of phytochemicals and evaluation of anti- cancer potential of Blumea eriantha DC. Physiology and Molecular Biology of Plants: An International Journal of Functional Plant Biology, 20(4), 475–486. https://doi.org/10.1007/s12298-014-0246-2

Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994;344:721-4.

Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 2nd ed. Oxford, UK: Oxford University Press, 1989.

Halliwell B, Aruoma 01. DNA and free radicals. Chichester, UK: Ellis-Harwood, 1993.

Han Q, Song J, Qiao C, Wong L, Xu H. J.Sep. Sci. 2006, 29, 1653-1657

Harris, I. S., Treloar, A. E., Inoue, S., Sasaki, M., Gorrini, C., Lee, K. C., … Mak, T. W. (2015). Glutathione and Thioredoxin Antioxidant Pathways Synergize to Drive Cancer Initiation and Progression. Cancer Cell, 27(2), 211–222. https://doi.org/10.1016/J.CCELL.2014.11.019

Hussain, S. A. (2013). Comprehensive update on cancer scenario of Bangladesh. South Asian Journal of Cancer, 2(4), 279. https://doi.org/10.4103/2278-330X.119901

Jagetia GC, Baliga MS, Malagi KJ, Kamath MS. The 25. evaluation of the radioprotective effect of Triphala (an Ayurvedic rejuvenating drug) in the mice exposed to gamma-radiation. Phytomedicine 2002;9:99-108.

Jagetia GC, Malagi KJ, Baliga MS, Venkatesh P, Veruva 26. RR. Triphala an

Ayurvedic rasayana drug protects mice against radiation-induced lethality by free-radical scavenging. J Altern Complement Med 2004;10:971-978.

Juang LJ, Sheu SJ, Lin TC,. J.Sep. Sci. 2004, 27, 718-724. 24.

Kabidul Azam, M. N., Rahman, M. M., Biswas, S., & Ahmed, M. N. (2016). Appraisals of Bangladeshi Medicinal Plants Used by Folk Medicine Practitioners in the Prevention and Management of Malignant Neoplastic Diseases. International Scholarly Research Notices, 2016, 1–12. https://doi.org/10.1155/2016/7832120

Kaur S, Michael H, Arora S, Härkönen PL, Kumar S. The 21. in vitro cytotoxic and apoptotic activity of Triphala–an Indian herbal drug. J Ethnopharmacol 2005;97:15-20.

Khatri, S., Phougat, N., Chaudhary, R., Singh, B., & Chhillar, A. K. (2016). International journal of pharmacy and pharmaceutical sciences. International Journal of Pharmacy and Pharmaceutical Sciences (Vol. 8). IJPPS. Retrieved from https://innovareacademics.in/journals/index.php/ijpps/article/view/12637

Klein, G. (2009). Toward a genetics of cancer resistance. Proceedings of the National Academy of Sciences of the United States of America, 106(3), 859–863. https://doi.org/10.1073/pnas.0811616106

Kumar Sampath KP. Recent trends in potential traditional Indian herbs Emblica officinalis and its medicinal importance. Journal of Pharmacognosy and Phytochemistry. 2012; 1(1):18-28.

Kumudhavalli MV, Vyas Mohit and Jayakar B. Phytochemical and Pharmacological evalution of the plant fruit of Terminalia belerica Roxb, International Journal of Pharmacy and Life Sciences. 2010;1(1):1-11.

Lalhminghlui, K., & Jagetia, G. C. (2018). Evaluation of the free-radical scavenging and antioxidant activities of Chilauni, Schima wallichii Korth in vitro. Future Science OA, 4(2), FSO272. https://doi.org/10.4155/fsoa-2017-0086

Li, Y., Xu, C., Zhang, Q., Liu, J. Y., & Tan, R. X. (2005). In vitro anti-Helicobacter pylori action of 30 Chinese herbal medicines used to treat ulcer diseases. Journal of Ethnopharmacology, 98(3), 329–333. https://doi.org/10.1016/j.jep.2005.01.020

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy Reviews, 4(8), 118–126. https://doi.org/10.4103/0973-7847.70902

Lu, H., Ouyang, W., & Huang, C. (2006). Inflammation, a key event in cancer development. Molecular Cancer Research: MCR, 4(4), 221–233. https://doi.org/10.1158/1541- 7786.MCR-05-0261

Lulekal, E., Kelbessa, E., Bekele, T., & Yineger, H. (2008). An ethnobotanical study of medicinal plants in Mana Angetu District, southeastern Ethiopia. Journal of Ethnobiology and Ethnomedicine, 4(1), 10. https://doi.org/10.1186/1746-4269-4-10

Mans, D. R. A. (2004). Anti-Cancer Drug Discovery and Development in Brazil: Targeted Plant Collection as a Rational Strategy to Acquire Candidate Anti-Cancer Compounds. The Oncologist, 5(3), 185–198. https://doi.org/10.1634/theoncologist.5-3-185

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clinical Science (London, England :

1979), 84(4), 407–412. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8482045

Nadkarni KM. Indian Meteria Medica, Published by Ramdas Bhatkal for Popular Prakashan Pvt. Ltd. Mumbai.2002; 01:2021205.

Nariya M, Shukla V, Jain S, Ravishankar B. Comparison of 28. enteroprotective efficacy of Triphala formulations (Indian Herbal Drug) on methotrexate-induced small intestinal damage in rats. Phytother Res 2009;23:1092-1098.

Nariya M, Shukla V, Jain S, Ravishankar B. Comparison of 28. enteroprotective efficacy of Triphala formulations (Indian Herbal Drug) on methotrexate-induced small intestinal damage in rats. Phytother Res 2009;23:1092-1098.

Ogura, S., & Shimosawa, T. (2014). Oxidative Stress and Organ Damages. Current Hypertension Reports, 16(8), 452. https://doi.org/10.1007/s11906-014-0452-x

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005). Global Cancer Statistics, 2002. CA: A Cancer Journal for Clinicians, 55(2), 74–108. https://doi.org/10.3322/canjclin.55.2.74

P. P., Patawar, V. A. (2012). Phytochemistryical & Anthelmintic Studies on Triphala. Indo Global Journal of Pharmaceutical Sciences (Vol. 2). Retrieved from https://pdfs.semanticscholar.org/11db/478740a67fff9738cc9fb4a472478cbcc5bc.pdf

Prakash Chandra Gupta, Biological and Pharmacological Properties of Terminalia Chebula Retz. Haritaki)- An Overview, International Journal of Pharmacy And Pharmaceutical Sciences 2012, 4(3).

Rebaya, A., Igueld Belghith, S., Baghdikian, B., Mahiou Leddet, V., Mabrouki, F., Olivier, E., Trabelsi Ayadi, M. (2014). Total Phenolic, Total Flavonoid, Tannin Content, and Antioxidant Capacity of Halimium halimifolium (Cistaceae) ARTICLE INFO ABSTRACT. Journal of Applied Pharmaceutical Science, 5(01), 52-057. https://doi.org/10.7324/JAPS.2015.50110

Rosenbluth, K. H., Gimenez, F., Kells, A. P., Salegio, E. A., Mittermeyer, G. M., Modera, K.,Bankiewicz, K. S. (2013). Automated segmentation tool for brain infusions. PloS One, 8(6), e64452. https://doi.org/10.1371/journal.pone.0064452

Sabina EP, Rasool M. Analgesic antipyretic and ulcerogenic 15. effects of Indian Ayurvedic herbal formulation Triphala. Res J Med Plant 2007;1:54-59.

Sabu MC, Kuttan R. Antidiabetic activity of medicinal 18. plants and its relationship with their antioxidant property. J Ethnopharmacol 2002;81:155-160

Sailaja Rao, P., Kalva, S., Yerramilli, A., & Mamidi, S. (2012). Free Radicals and Tissue Damage: Role of Antioxidants. Free Radicals and Antioxidants, 1(4), 2–7. https://doi.org/10.5530/ax.2011.4.2

Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., & Longacre, M. (2013).
Cancer development, progression, and therapy: an epigenetic overview. International Journal of Molecular Sciences, 14(10),21087–21113. https://doi.org/10.3390/ijms141021087

Sandhya T, Lathika KM, Pandey BN, Bhilwade HN, 27. Chaubey RC, Priyadarsini KI, et al. Protection against radiation oxidative damage in mice by Triphala. Mutat Res2006;609:17-25.

Sandhya T, Lathika KM, Pandey BN, Mishra KP. Potential 22. of traditional Ayurvedic formulation Triphala as a novel anticancer drug. Cancer Lett 2006;231:206-214.

Sandhya T, Mishra KP. Cytotoxic response of breast cancer 23. cell lines MCF-7 and T47D to Triphala and its modification by antioxidants. Cancer Lett 2006;238:304-313

Satyal, P., Chhetri, B. K., Dosoky, N. S., Shrestha, S., Poudel, A., & Setzer, W. N. (n.d.). Chemical Composition of Triphala Essential Oil from Nepal. Biological Activities of the Essential Oil and (Z)-Lachnophyllum Ester. Retrieved from http://www.tropicos.org/Name/2700314;

Scheck, A. C., Perry, K., Hank, N. C., & Clark, W. D. (2006). Anticancer activity of extracts derived from the mature roots of Scutellaria baicalensis on human malignant brain tumor cells. BMC Complementary and Alternative Medicine, 6(1), 27. https://doi.org/10.1186/1472-6882-6-27

Seca AM, Pinto DC. Plant secondary metabolites as anticancer agents: Successes in clinical trials and therapeutic application. International Journal of Molecular Sciences. 2018;19(263):1-22

Senthilraja, P., & Kathiresan, K. (2015). In vitro cytotoxicity MTT assay in Vero, HepG2 and MCF-7 cell lines study of Marine Yeast ARTICLE INFO ABSTRACT. Journal of Applied Pharmaceutical Science, 5(03), 80-084.https://doi.org/10.7324/JAPS.2015.50313

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. Journal of Botany, 2012, 1–26.

https://doi.org/10.1155/2012/217037

Shi Y, Sahu RP, Srivastava SK. Triphala inhibits both 24. in vitro and in vivo xenograft growth of pancreatic tumor cells by inducing apoptosis. BMC Cancer 2008;8:294-235.

Singh V and Singh HK, Studies on Processing of Aonla (EmblicaphyllanthusGarten.) Fruits, Beverage and Food World, 2002.

Sofowora, A., Ogunbodede, E., & Onayade, A. (2013). The role and place of medicinal plants in the strategies for disease prevention. African Journal of Traditional, Complementary, and Alternative Medicines: AJTCAM, 10(5), 210–229. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24311829

Srikumar R, Parthasarathy NJ, Shankar EM, Manikandan 6. S, Vijayakumar R, Thangaraj R, et al. Evaluation of the growth inhibitory activities of Triphala against common bacterial isolates from HIV infected patients. Phyto Res 2007;21:476-480.

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., & Hawkins Byrne, D. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. Journal of Food Composition and Analysis, 19(6–7), 669–675. https://doi.org/10.1016/J.JFCA.2006.01.003

Triphala(2020). Retried from https://www.banyanbotanicals.com/info/plants/ayurvedic-herbs/triphala/

Troup, S. B., Swisher, S. N., & Young, L. E. (1960). The anemia of leukemia. The American Journal of Medicine, 28(5), 751–763. https://doi.org/10.1016/0002-9343(60)90132-7

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. Nutrients, 2(12), 1231– 1246. https://doi.org/10.3390/nu2121231

Vadde, R., Radhakrishnan, S., Reddivari, L., & Vanamala, J. (2015). Triphala Extract Suppresses Proliferation and Induces Apoptosis in Human Colon Cancer Stem Cells via Suppressing c-Myc/Cyclin D1 and Elevation of Bax/Bcl-2 Ratio. Biomed Research International, 2015, 1-12. doi: 10.1155/2015/649263

Wang, B.-Q. (2010). Salvia miltiorrhiza: Chemical and pharmacological review of a medicinal plant. Journal of Medicinal Plants Research, 4(25), 2813–2820. Retrieved from http://www.academicjournals.org/JMPR

Wink, M. (2015). Modes of Action of Herbal Medicines and Plant Secondary Metabolites. Medicines, 2(3), 251–286. https://doi.org/10.3390/medicines2030251

Wu, J. Q., Kosten, T. R., & Zhang, X. Y. (2013). Free radicals, antioxidant defense systems, and schizophrenia. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 46, 200–206. https://doi.org/10.1016/j.pnpbp.2013.02.015

Young, I. S., & Woodside, J. V. (2001). Antioxidants in health and disease. Journal of Clinical Pathology, 54(3), 176–186. https://doi.org/10.1136/JCP.54.3.176