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“In Vitro Antioxidant Activity and Phytochemical Characterization of *Hedyotis corymbosa* (L.) Lam. Using Multiple Solvent Extracts”

A Dissertation submitted to Department of Pharmacy, Independent University Bangladesh in partial fulfilment of the requirements for the degree of Bachelor of Pharmacy

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Abstract

Hedyotis corymbosa (L.) Lam. (Rubiaceae) is a small annual herb widely used in traditional medicine systems across South and Southeast Asia for the treatment of fever, jaundice, liver disorders, and inflammatory conditions. Despite its established ethnobotanical significance, a comprehensive evaluation of its antioxidant potential across multiple solvent fractions has not been previously reported for plant material sourced from Bangladesh. The present study aimed to characterise the antioxidant activity of n-hexane, chloroform, and crude methanol extracts of the whole plant through a panel of seven complementary in vitro assays.

Extraction was performed by maceration, yielding 3.6g, 17.6g, and 23.3g for the n-hexane, chloroform, and methanol fractions respectively. Total phenolic content (TPC) and total flavonoid content (TFC) were highest in the chloroform extract (47.75 mg GAE/g and 32.85 mg QE/g), followed by the methanol extract, and lowest in the n-hexane fraction. Antioxidant activity was evaluated through DPPH radical scavenging, hydroxyl radical scavenging (HRSA), total antioxidant capacity (TAC), ferric reducing antioxidant power (FRAP), and lipid peroxidation inhibition assays. The chloroform extract showed slightly better %I results than the methanol extract while the n-hexane extract showed the weakest activity across all assays.

These findings confirm that *H. corymbosa* possesses significant antioxidant potential, with flavonoids and phenolic compounds identified as the primary bioactive constituents. The results provide scientific support for the plant's traditional medicinal uses and establish a phytochemical basis for further pharmacological investigation.

Table of Contents

Abstract 2

Chapter 1: Introduction 7

1.1 The Study Plant: Hedyotis corymbosa (L.) Lam. 11

1.1.1 Taxonomic Classification and Geographical Distribution 11

1.1.2 Previously Reported Phytochemistry and Biological Activity 11

1.2 Rationale for Solvent Selection: The Role of Polarity in Phytochemical Extraction 12

1.3 Justification for the Present Study 13

1.4 Objectives of the Study 14

1.5 Scope and Limitations of the Study 15

Chapter 2: Literature Review 17

2.1 Oxidative Stress and Free Radicals 17

2.1.1 Oxidative Stress, Free Radicals, and Neurodegeneration 23

2.2 Plant Secondary Metabolites as Natural Antioxidants 28

2.3 Overview of Antioxidant Assays Employed 29

2.3.1 DPPH Radical Scavenging Assay 30

2.3.2 Total Antioxidant Capacity (TAC) — Phosphomolybdenum Method 30

2.3.3 Hydroxyl Radical Scavenging Activity (HRSA) 31

2.3.4 Total Phenolic Content (TPC) — Folin-Ciocalteu Method 31

2.3.5 Total Flavonoid Content (TFC) 32

2.3.6 Lipid Peroxidation Inhibition 32

2.3.7 Ferric Reducing Antioxidant Power (FRAP) 33

Chapter 3: Materials and Method 34

3.1 Plant Material 34

3.2 Preparation of Extracts 34

3.2.1 Primary Methanol Extraction 34

3.2.2 Liquid-Liquid Fractionation: n-Hexane Fraction 35

3.2.3 Liquid-Liquid Fractionation: Chloroform Fraction 36

3.2.4 Summary of Extract Yields 37
3.3 Chemicals and Reagents 37
3.4 Phytochemical Screening and Antioxidant properties 38
3.5 Determination of Total Phenolic Content (TPC) 38
3.6 Determination of Total Flavonoid Content (TFC) 40
3.7 Determination of Total Antioxidant Capacity (TAC) 42
3.8 DPPH Free Radical Scavenging Activity 43
3.9 Hydroxyl Radical Scavenging Activity (HRSA) 45
3.10 Lipid Peroxidation Inhibition Assay 47
3.11 Ferric Reducing Antioxidant Power (FRAP) Assay 49
Chapter 4: Result 51
4.1 Total Phenolic Content (TPC) 51
4.2 Total flavonoid content (TFC) 54
4.3 DPPH free radical scavenging assay 57
4.4 Hydroxyl radical scavenging assay 62
4.5 Total Antioxidant Capacity (TAC) 68
4.6 Ferric reducing antioxidant power assay (FRAP) 73
4.7 Lipid Peroxidation Inhibition Test 78
Chapter 5: Discussion 84
5.1 TPC result interpretation 84
5.2 TFC result interpretation 86
5.3 DPPH result interpretation 88
5.4 HRSA result interpretation 89
5.5 TAC result interpretation 91
5.6 FRAP assay result interpretation 92
5.7 Lipid peroxidation inhibition assay result interpretation 94
Chapter 6: Conclusion 98
Reference **101**

List of Figures

Figure 1: Standard curve of Gallic acid..... 52
Figure 2: Comparison of mean TPC of methanol, chloroform and n-hexane extracts 54
Figure 3: Standard curve of quercetin 55
Figure 4: Comparison between TFC values of methanol, chloroform and n-hexane extract 57
Figure 5: DPPH assay result of Standard, methanol, chloroform and n-Hexane extract 62
Figure 6: HRSA result comparison between standard, methanol, chloroform and n-Hexane extract 68
Figure 7: Comparison between test results of standard ascorbic acid, methanol, chloroform and n-hexane extract for TAC..... 73
Figure 8: Comparison between test results of FRAP amongst ascorbic acid, methanol, chloroform, n-Hexane extract 78
Figure 9: Comparison between standard gallic acid, methanol, chloroform, n-hexane extract for Lipid peroxidation inhibition 83

List of Tables

Table 1: Summary of extract yields 37
Table 2: Gallic acid calibration curve absorbance values (760 nm) used for TPC calculation ... 51
Table 3: Mean TPC of crude methanol extract 52
Table 4: Mean TPC of chloroform extract 53
Table 5: Mean TPC of n-Hexane extract..... 53
Table 6: Qquercetin absorbance to make standard curve for the calculation of TFC 55
Table 7: Methanol extract absorbance and mean TFC..... 56
Table 8: Chloroform extract absorbance and mean TFC 56
Table 9: n-Hexane extraction absorbance and mean TFC 56
Table 10: Standard (ascorbic acid) absorbance and %I value for DPPH assay 58
Table 11: Methanol extract DPPH assay result..... 59
Table 12: Chloroform extract DPPH assay result 60
Table 13: n-Hexane extract DPPH assay result 61
Table 14: Ascorbic acid result for HRSA 64
Table 15: Methanol extract result for HRSA assay..... 65

Table 16: Chloroform extract resulf for HRSA 66
Table 17: n-Hexane extract result for HRSA 67
Table 18: Ascorbic acid (Standard) results for TAC 69
Table 19: Methanol extract test result for TAC 70
Table 20: Chloroform extract test result for TAC 71
Table 21: n-Hexane test result for TAC 72
Table 22: Ascorbic acid test result for FRAP 74
Table 23: Methanol extract test result for FRAP 75
Table 24: Chloroform extract test result for FRAP 76
Table 25: n-Hexane extract test result for FRAP 77
Table 26: Ascorbic acid test resulf for Lipid peroxidation Inhibition 79
Table 27: Methanol extract test result for Lipid peroxidation inhibition 80
Table 28: Chloroform extract test result for Lipid peroxidation inhibition 81
Table 29: n-Hexane extraction test result for Lipid peroxidation inhibition 82

List of Abbreviations

WHO	World Health Organization
AD	Alzheimer's Disease
PD	Parkinson's Disease
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
CAT	Catalase
GPx	Glutathione peroxidase
BHT	Butylated Hydroxytoluene
H.*corymbosa*	Hedyotis corymbosa
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
TAC	Total Antioxidant Capacity
FRAP	Ferric Reducing Antioxidant Power Assay
ETC	Electron Transport Chain
GSH	Glutathione
GSSH	Glutathione disulfide
L•	Lipid radical
•OH	Hydroxyl radical
LOOH	Lipid hydroperoxide
LO•	Lipid alkoxy radical
LOO•	Lipid peroxyl radical
MDA	Malondialdehyde
4-HNE	4-hydroxynonenal
8-OHdG	8-hydroxy-2'-deoxyguanosine
CNS	Central nervous system
PUFA	Polyunsaturated fatty acid
Aβ	Amyloid-β
NFTs	Neurofibrillary tangles
SNpc	Substantia nigra pars compacta
DPPH	2,2-diphenyl-1-picrylhydrazyl
HRSA	Hydroxyl Radical Scavenging Assay
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
PMF	Polymethoxylated flavonoids
HAT	Hydrogen atom transfer
SET	Single electron transfer

Chapter 1: Introduction

Plants have played the role of the foundation of human medicine for thousands of years. Long before synthetic pharmaceuticals were developed, communities across Asia, Africa, and the Americas relied on plant-derived concoction to manage pain, treat infections, and restore health. Even today, a considerable segment of the global population, particularly in rural settings continues to depend on medicinal plants as a primary healthcare resource. The World Health Organization (WHO) has estimated that more than 80% of the global population relies on traditional plant-based medicine to meet at least part of their basic healthcare needs(Organization & others, 2013). This relationship between humans and plants is not merely cultural, it reflects the real pharmacological activity of thousands of naturally occurring compounds that modern science has only begun to characterize in a systematic and rigorous way.

The global burden of non-communicable disease has intensified interest in plant-derived therapeutics. Cardiovascular disease, type 2 diabetes mellitus, hepatocellular disorders, and neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD) together account for a disproportionate share of worldwide morbidity and mortality(Sies, 2015). A common biochemical feature shared across this spectrum of diseases is oxidative stress, the condition in which the intracellular generation of reactive oxygen species (ROS) exceeds the capacity of antioxidant defense systems to neutralize them, resulting in progressive oxidative damage to lipids, proteins, and nucleic acids(Lobo et al., 2010).

One of the most extensively investigated aspects of medicinal plant research in recent decades has been antioxidant activity. Interest in plant antioxidants has been driven largely by the growing scientific understanding that ROS, including superoxide anion radicals (O•2•−), hydroxyl radicals (•OH), and non-radical species such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) play pivotal roles in the pathogenesis of a broad spectrum of diseases(Valko et al., 2007). When the balance between ROS generation and antioxidant defense is disrupted due to environmental pollutants, poor diet, chronic inflammation, or aging, the resulting oxidative damage can affect DNA integrity, protein function, and membrane lipid composition, contributing to cancer, cardiovascular disorders, diabetes mellitus, and neurodegenerative conditions(Lobo et al., 2010; Valko et al., 2007).

The human body does possess its own antioxidant defense mechanisms, including enzymatic systems such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as non-enzymatic antioxidants including vitamins C and E, glutathione, and uric acid(Halliwell & Gutteridge, 2015). However, these endogenous systems are not always sufficient to cope with chronically elevated oxidative loads, and dietary antioxidants from plant sources play an important complementary and synergistic role in maintaining redox homeostasis(Scalbert & Williamson, 2000). Plants synthesize a large number of secondary metabolites, compounds not directly involved in primary growth and development, but serving key ecological functions such as protection against herbivores, UV radiation, and microbial attack. Many of these metabolites, including polyphenols, flavonoids, terpenoids, and alkaloids, are also potent antioxidants whose

structural features allow them to donate electrons or hydrogen atoms to free radicals, effectively neutralizing them before they cause biological harm(Rice-Evans et al., 1996).

There is also increasing regulatory and commercial pressure to replace synthetic food antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) with natural plant-derived alternatives, following safety concerns raised by long-term toxicological studies suggesting carcinogenic and endocrine-disrupting potential(Shahidi & Zhong, 2010). This has started a global effort to systematically identify and characterize plant species whose antioxidant potential has not yet been rigorously validated under controlled laboratory conditions.

It is within this scientific context that the present study focuses on Hedyotis corymbosa (L.) Lam., a small annual herb belonging to the family Rubiaceae. It's antioxidant properties have not been comprehensively characterized using a systematic, multi-solvent, multi-assay approach. This study therefore prepares crude extracts of H. corymbosa using three solvents of differing polarity, n-hexane, chloroform, and methanol that evaluates their antioxidant activity through a panel of seven antioxidant assays: DPPH radical scavenging, total antioxidant capacity, hydroxyl radical scavenging, total phenolic content, total flavonoid content, lipid peroxidation inhibition, and ferric reducing capacity.

1.1 The Study Plant: Hedyotis corymbosa (L.) Lam.

1.1.1 Taxonomic Classification and Geographical Distribution

Hedyotis corymbosa (L.) Lam., also known under its synonym Oldenlandia corymbosa L., is a small erect annual herb belonging to the family Rubiaceae (Panta, 2022). The plant is characterized by slender, quadrangular stems, narrow lanceolate to linear leaves with prominent interpetiolar stipules, and small white to pale pink flowers arranged in axillary or terminal cymose clusters. It typically attains a height of 10–40 cm and is commonly found in open, disturbed habitats including roadsides, rice paddies, waste grounds, and fallow agricultural fields(Kirtikar, 1991). In Bangladesh, the plant is widely distributed in rural areas throughout the country and is known by various vernacular names in regional dialects. Taxonomically, the synonymy between H. corymbosa and O. corymbosa reflects historical revisions within the tribe Spermacoceae of the subfamily Rubioideae; the accepted name Hedyotis corymbosa (L.) Lam. is used consistently throughout this thesis.

1.1.2 Previously Reported Phytochemistry and Biological Activity

Phytochemical investigations of H. corymbosa have identified a range of secondary metabolites. Iridoid glycosides, a class of compounds characteristic of the Rubiaceae family have been isolated, including asperuloside and scandoside methyl ester(Lin et al., 1995). Qualitative phytochemical screening studies have consistently reported the presence of alkaloids, saponins, tannins, sterols, and terpenoids in varying concentrations depending on the solvent used and the geographic source of the plant material(Kirtikar, 1991). A systematic and comprehensive multi-solvent, multi-assay evaluation of antioxidant properties has not previously been reported for plant material from Bangladesh, a gap that the present study addresses.

1.2 Rationale for Solvent Selection: The Role of Polarity in Phytochemical Extraction

The choice of extraction solvent is a critical determinant of which classes of phytochemicals are recovered from plant material, and therefore of which biological activities are captured and quantified in the resulting extract. The fundamental guiding principle that like dissolves like, meaning solvent dissolves compounds of similar polarity dictating that a single-solvent extraction will capture only a fraction of the total phytochemical diversity present in a plant(Ncube et al., 2008). Sequential solvent extraction using solvents of progressively increasing polarity allows systematic fractionation of the total phytochemical contents into chemically distinct fractions, each enriched for compound classes of corresponding polarity, and is therefore the approach most recommended by phytochemical research guidelines for comprehensive antioxidant evaluation(Tiwari et al., 2011).

n-Hexane is a non-polar aliphatic hydrocarbon solvent (dielectric constant $\varepsilon \approx 1.9$) that selectively dissolves lipophilic compounds such as fixed oils, waxes, long-chain fatty acids, chlorophylls, carotenoids, and non-polar terpenoids and sterols(Harborne, 1998). The n-hexane extract is therefore enriched with the lipophilic or "fat-soluble" fraction of the plant. Although this fraction is generally not expected to be rich in phenolics or flavonoids which are relatively polar, it may still exhibit antioxidant activity (Decker, 1998).

Chloroform is a semi-polar solvent (ε ≈ 4.8) capable of dissolving compounds with intermediate polarity, including many alkaloids in their free base form, aglycone flavonoids, non-polar terpenoids and steroids, and glycosides with significant lipophilic character(Harborne, 1998). The chloroform fraction therefore occupies a chemical middle ground between the lipophilic hexane fraction and the highly polar methanol fraction, and often contains biologically active compounds not efficiently recovered by either of the other two solvents alone(Cowan, 1999).

Methanol is a polar protic solvent (ε ≈ 32.7) and is generally the most effective solvent for extracting the broadest and most abundant range of phenolic compounds, including phenolic acids, flavonoid glycosides, tannins, anthocyanins, and other polar secondary metabolites(Azwanida, 2015). It is by far the most widely used solvent in antioxidant screening studies of medicinal plants, consistently yielding the highest total phenolic content and the strongest antioxidant activity in comparative extraction studies(Spigno et al., 2007). In the present study, data from all three fractions were evaluated and compared to understand the contribution of different compound classes to overall antioxidant capacity.

1.3 Justification for the Present Study

Despite its documented ethnobotanical significance and preliminary pharmacological activity, H.*corymbosa* has not been subjected to a comprehensive, multi-solvent, multi-assay antioxidant evaluation using plant material sourced from Bangladesh. Local environmental factors including soil composition, humidity, temperature, altitude, and

seasonal variation significantly influence the secondary metabolite profiles of plants, and findings from studies conducted on the same species in India, China, or Nigeria cannot therefore be assumed to reflect the phytochemical composition or pharmacological activity of Bangladeshi specimens(Gobbo-Neto & Lopes, 2007). Locally generated data are therefore scientifically and practically important for informing both research priorities and potential applications of this plant in the Bangladeshi context.

Furthermore, the use of three solvents of differing polarity in the present study enables a more nuanced understanding of which chemical fractions carry the most antioxidant activity, and the use of seven complementary assays addresses the mechanistic breadth of that activity. This level of characterization is necessary if H. corymbosa is to be seriously considered as a candidate for development as a natural antioxidant source for pharmaceutical, nutraceutical, or cosmetic applications, all sectors that currently face regulatory and consumer pressure to reduce dependence on synthetic antioxidant additives(Buer et al., 2010).

1.4 Objectives of the Study

The present study was designed to provide a systematic and comprehensive evaluation of the antioxidant potential of *Hedyotis corymbosa* (L.) Lam. through a multi-extract, multi-assay approach. The first objective was to prepare crude solvent extracts of the plant using n-hexane, chloroform, and methanol and to calculate the percentage yield of each extract as a basic indicator of extractability across different solvent polarities.

Following extraction, the total phenolic content (TPC) and total flavonoid content (TFC) of each extract were quantified using standard colorimetric methods, providing a concrete measure of polyphenolic richness across the three fractions.

The study then proceeded to evaluate antioxidant activity through a panel of five mechanistically distinct in vitro assays. Free radical scavenging capacity was assessed using the DPPH assay and the hydroxyl radical scavenging activity (HRSA) assay. The overall electron-donating and reductive capacity of the extracts was further characterized through the total antioxidant capacity (TAC) assay, and the ferric reducing antioxidant power (FRAP) assay.

Finally, the antioxidant activities of the three extracts were compared against standard reference antioxidants and the relationship between phytochemical content (TPC and TFC) and the measured antioxidant activities was analysed, with the aim of identifying which compound classes are the primary drivers of antioxidant capacity in this species and which solvent fraction represents the most pharmacologically promising source of natural antioxidants from *H. corymbosa*.

1.5 Scope and Limitations of the Study

This study is confined to in vitro antioxidant evaluation methods. While in vitro assays constitute a well-established and widely accepted first step in the screening of natural

product antioxidant activity, they do not account for the pharmacokinetic variables that determine bioavailability in a living organism (Manach et al., 2005). The results of this study should therefore be interpreted as evidence of antioxidant potential, not confirmed therapeutic efficacy. In vivo studies using appropriate animal models, and ultimately clinical investigations in human subjects, would be necessary to establish pharmacological relevance and therapeutic safety.

Additionally, plant material was collected from a single geographic location at a single time point. Secondary metabolite profiles in plants are subject to intraspecific variation influenced by geographic provenance, phenological stage (developmental phase at time of collection), soil characteristics, altitude, and seasonal factors such as rainfall and temperature. The findings of this study are therefore representative of the specific sample analyzed and may not generalize fully to H. corymbosa populations from other regions of Bangladesh or other countries. Multi-site and multi-season studies would be required to generate a generalizable phytochemical and antioxidant profile for this species at the national level.

Chapter 2: Literature Review

2.1 Oxidative Stress and Free Radicals

Free radicals are atoms or molecules that contain one or more unpaired electrons in their outer orbital, a structural feature that renders them thermodynamically unstable and chemically highly reactive(Gutteridge & Halliwell, 1993). Free radicals are unstable because they carry an unpaired electron, and they try to become stable by stealing electrons from nearby molecules in the body. In doing so, they can damage a wide range of biological structures including the fatty acids that make up cell membranes, proteins that contain sulfur groups, and the sugar-and-base building blocks of DNA. The problem is that when a free radical takes an electron from one of these molecules, it often turns that molecule into a new radical, which then attacks another molecule, and so on. This creates a damaging chain reaction that, if the body cannot stop it in time, can cause serious and accumulating harm to cells and tissues (Halliwell, 2006). The term "reactive oxygen species" (ROS) encompasses not only true oxygen-centred free radicals such as superoxide (O•2•−), hydroxyl (•OH), and peroxyl radicals (ROO•), but also non-radical oxidizing intermediates capable of generating radicals in biological systems, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and peroxynitrite (ONOO−)(Sies & Jones, 2020). Reactive nitrogen species (RNS), principally nitric oxide (NO•) and its highly reactive derivative peroxynitrite, further contribute to the overall burden of oxidative and nitrosative stress in living systems, causing protein nitration at tyrosine residues and disruption of redox-sensitive cell-signalling networks(Pacher et al., 2007).

In biological systems, the superoxide radical (O•2•−) is typically the primary ROS generated, produced mainly as a byproduct of electron leakage from the mitochondrial electron transport chain (ETC) and by membrane-bound and cytosolic enzymes including NADPH, xanthine oxidase, and cytochrome P450 enzymes(Murphy, 2009). Under physiological conditions, superoxide is rapidly converted to the less reactive H_2O_2 by the metalloenzymes SOD(Fukai & Ushio-Fukai, 2011). Although H_2O_2 is not itself a radical, it is membrane-permeable and can react with reduced transition metal ions, particularly ferrous iron (Fe^{2+}) and cuprous copper (Cu^{+}), through the Fenton reaction ($Fe^{2+} + H^{2}02 \rightarrow Fe^{3+} + \bullet OH + OH^{-}$) to yield the hydroxyl radical (•OH), universally regarded as the most reactive and biologically damaging ROS(Liochev, 2013).

The Haber–Weiss reaction further amplifies hydroxyl radical generation by coupling the Fenton reaction to the superoxide-mediated reduction of Fe^{3+} back to Fe^{2+}, thereby perpetuating a self-sustaining cycle of radical generation that is particularly destructive in iron-rich biological compartments such as the mitochondrial matrix and the labile iron pool of neurons(Koppenol & Hider, 2019). Because no known enzymatic mechanism exists to directly and specifically scavenge hydroxyl radicals, the organism must rely on small-molecule, non-enzymatic antioxidants, including glutathione (GSH), ascorbic acid, α-tocopherol, and a broad range of plant-derived polyphenolic compounds to intercept them before they cause irreversible biological damage(Pham-Huy et al., 2008).

Oxidative stress is not merely defined by an excess of ROS, but rather by a sustained imbalance in which ROS are produced faster than the body's antioxidant defenses can neutralize them, a prolonged disruption that tips the scales toward cellular damage. Sies and colleagues, who pioneered this concept, further clarified that oxidative stress is not simply a matter of quantity; it also involves disruptions in the way cells use ROS as chemical signals. In other words, even subtle shifts in the normal redox balance can interfere with critical cell signaling processes, without there necessarily being a dramatic spike in overall ROS levels.(Sies, 2015). This distinction matters because not all ROS are harmful. At low, controlled levels, ROS actually serve important roles in keeping the body functioning normally. They act as chemical messengers that help cells communicate, regulate the activity of proteins that control gene expression, help maintain healthy blood vessel function, and assist immune cells in killing bacteria and other pathogens. So rather than being purely destructive, ROS are essential to normal biology, the problem only arises when their levels become excessive and uncontrolled.(D'Autréaux & Toledano, 2007).

The body has a built-in antioxidant defence system made up of several enzymes that work together in a coordinated chain. First, an enzyme called superoxide dismutase (SOD) converts the harmful superoxide radical into hydrogen peroxide (H_2O_2). Although hydrogen peroxide is less reactive, it is still damaging, so it must be broken down further. This is done by two other enzymes: CAT, which breaks H_2O_2 directly into harmless water and oxygen, and GPx , which neutralizes H_2O_2 by using a protective molecule called glutathione (GSH) — converting it in the process to its oxidized form, glutathione disulfide (GSSG). Together, these enzymes form an interlocking defence

network that keeps ROS levels in check. (GSSG)(Halliwell & Gutteridge, 2015). Glutathione reductase then restores GSH from GSSG at the expense of NADPH, linking the antioxidant defense network to the pentose phosphate pathway. Additional enzymatic antioxidants include thioredoxin reductase, peroxiredoxins, and glutaredoxins, which together constitute an interlocking redox relay system that maintains the reducing environment essential for normal cellular function(Sies & Jones, 2020).

One of the most well-studied and damaging consequences of uncontrolled ROS activity is lipid peroxidation, the oxidative breakdown of fats in cell membranes. This process unfolds in three stages: initiation, propagation, and termination(Yin et al., 2011).

In the initiation stage, a highly reactive free radical, most commonly the hydroxyl radical, attacks a fatty acid in the cell membrane, pulling away a hydrogen atom and creating an unstable fatty acid radical (L•). In the propagation stage, this unstable radical reacts with oxygen to form a lipid peroxyl radical (LOO•), which then attacks a neighboring fatty acid, creating yet another radical and a toxic byproduct called a lipid hydroperoxide (LOOH). This sets off a chain reaction that can damage thousands of fatty acid molecules from a single starting event. The lipid hydroperoxides formed are themselves unstable and, in the presence of metal ions like iron, break down further to generate additional radicals, amplifying the damage.

Termination occurs when two radicals collide and cancel each other out, or when a chain-breaking antioxidant such as vitamin E steps in, donating a hydrogen atom to stop the chain reaction. Notably, vitamin E can be recycled back to its active form by vitamin C or plant-derived polyphenols, allowing it to continue protecting cells(Yin et al., 2011).

When lipid peroxidation runs its course, it leaves behind toxic breakdown products, most notably malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These molecules are chemically reactive and cause further damage by latching onto other biological molecules. MDA, for instance, binds to proteins and DNA, forming abnormal cross-links between them. This cross-linking physically distorts the structure of these molecules, disrupting the cell's ability to read and copy its genetic information, and impairing the normal function of proteins. In this way, the damage from lipid peroxidation extends well beyond the cell membrane, spreading into the very machinery that keeps cells alive and functioning.(Esterbauer et al., 1991). Similarly, 4-HNE binds to specific building blocks of proteins, namely cysteine, histidine, and lysine, and in doing so, disables several important enzymes involved in energy production and waste disposal within the cell. When these enzymes stop working properly, the cell struggles to generate energy and clear out damaged proteins, compounding the overall harm. Elevated levels of both MDA and 4-HNE have been detected in the blood and tissues of patients with a wide range of diseases driven by oxidative damage, including heart disease, fatty liver disease, type 2 diabetes, and various neurodegenerative conditions. This makes them useful measurable indicators or biomarkers that researchers and clinicians can use to assess the degree of oxidative damage occurring in the body (Dalle-Donne, Rossi, et al., 2006).

Beyond lipid peroxidation, oxidative damage to proteins, broadly termed oxidative protein modification, constitutes a second major pathway of ROS-induced cellular injury. •OH and peroxynitrite attack amino acid side chains, particularly those of methionine, cysteine, proline, arginine, lysine, and threonine. Protein carbonylation, the introduction of aldehyde or ketone groups into protein side chains, is now widely used as a global biomarker of protein oxidative damage, detectable by 2,4-dinitrophenylhydrazine (DNPH) derivatization followed by immunoblot or ELISA(Dalle-Donne, Aldini, et al., 2006). Oxidatively carbonylated proteins show altered conformation, impaired enzymatic activity, and increased susceptibility to aggregation, and are preferentially targeted for degradation by the 20S proteasome; however, when the proteasomal capacity is overwhelmed by an excessive burden of oxidized proteins, carbonylated proteins accumulate and form insoluble aggregates that are histopathological hallmarks of several neurodegenerative diseases(Dalle-Donne, Aldini, et al., 2006).

Oxidative damage to DNA is equally harmful for cellular viability and genomic integrity. Among the four DNA bases, guanine is the most vulnerable to attack by free radicals. When a hydroxyl radical strikes guanine at a specific position (carbon-8), it produces a damaged molecule called 8-hydroxy-2'-deoxyguanosine, or 8-OHdG for short. This modified molecule is now the most widely used and well-validated marker for detecting oxidative DNA damage in both laboratory research and clinical studies.(Cooke et al., 2003).

What makes 8-OHdG particularly dangerous is that it causes errors during DNA replication. Instead of pairing with its correct partner base (cytosine), it mistakenly pairs

with adenine, introducing a permanent mutation into the DNA sequence. If these mutations occur in genes that normally suppress tumour growth or regulate cell division, they can contribute to the development of cancer.

Beyond this, ROS cause several other forms of DNA damage, including breaks in the DNA strand, loss of individual bases, and abnormal bonds forming between DNA strands or between DNA and proteins. The cell detects these injuries and activates emergency repair pathways, triggering a cascade of molecular signals designed to halt cell division and fix the damage. However, when the damage is too extensive or persistent to be repaired, the cell is forced into one of two outcomes: it either enters a state of permanent growth arrest (senescence), or it undergoes programmed cell death (apoptosis)(Cooke et al., 2003).

2.1.1 Oxidative Stress, Free Radicals, and Neurodegeneration

The central nervous system (CNS) is exceptionally and inherently vulnerable to oxidative damage, a susceptibility arising from a constellation of structural, metabolic, and biochemical characteristics that distinguish neuronal tissue from most other organ systems. Although the human brain constitutes only approximately 2% of total body mass, it accounts for roughly 20% of total resting oxygen consumption and receives approximately 15% of cardiac output, making neuronal mitochondria extraordinarily prolific sources of superoxide as a byproduct of high-flux oxidative phosphorylation(Magistretti & Allaman, 2015). This disproportionate oxygen demand means that even small fractional increases in mitochondrial electron leakage, such as those caused by aging or genetic mutations in ETC subunit genes, translate into substantial increases in absolute ROS production within neuronal compartments.

Several additional features of neural tissue further compound its oxidative vulnerability. Neuronal plasma membranes and myelin sheaths are exceptionally enriched in PUFAs which, by virtue of their multiple double bonds, present multiple bisallylic hydrogen atoms vulnerable to radical abstraction, making them highly susceptible to lipid peroxidation chain reactions(Uttara et al., 2009). On top of this, the brain has surprisingly low levels of the antioxidant enzymes catalase and GPx compared to other organs like the liver. This means the brain is less well-equipped to break down hydrogen peroxide, leaving it more exposed to further radical damage. The brain also accumulates significant amounts of metal ions, particularly iron, copper, and manganese in key regions such as the substantia nigra, hippocampus, cortex, and striatum. These metals are chemically reactive and, when present alongside hydrogen peroxide, trigger reactions that generate the highly destructive hydroxyl radical directly within brain tissue. This is especially problematic because these reactions occur right where brain cells are most sensitive to oxidative damage(Zecca et al., 2004). Finally, neurons are largely post-mitotic cells with severely restricted regenerative capacity; unlike hepatocytes or haematopoietic cells, lost neurons are not replaced, and oxidatively damaged neurons that undergo apoptosis represent permanent and irreversible losses of functional neuronal circuitry.

The connection between oxidative stress and neurodegeneration is now well established across a number of distinct brain diseases. In Alzheimer's disease (AD) the most common neurodegenerative disorder, affecting over 55 million people worldwide, oxidative stress is now understood to play a dual role: it helps trigger the disease in the first place, and then continues to worsen it as the disease progresses. This damage is

closely tied to the two defining features of AD: the build-up of sticky protein clumps called amyloid-β (Aβ) plaques outside brain cells, and the formation of twisted protein threads known as neurofibrillary tangles (NFTs) inside them, made up of a damaged protein called tau(Cheignon et al., 2018). Amyloid-β peptides, particularly the most harmful form known as Aβ42 are not just passive bystanders in this process. They actively generate harmful free radicals, including hydrogen peroxide and superoxide, by interacting with metal ions like copper and iron that are bound to them. To make matters worse, these same free radicals then chemically damage the amyloid-β protein itself, causing it to misfold and clump together more rapidly. This creates a dangerous cycle: amyloid-β produces free radicals, those free radicals accelerate amyloid-β aggregation, and more aggregation leads to even more free radical production, each step feeding into and worsening the next.

Post-mortem analyses of AD brain tissue have consistently demonstrated significantly elevated levels of multiple oxidative damage biomarkers, including protein carbonyls, 4-HNE-modified proteins, 8-OHdG, and $F^2$2-isoprostanes in the hippocampus, entorhinal cortex, and inferior parietal lobule, the regions most selectively and severely affected by early AD neuropathology(Markesbery, 1997).

In Parkinson's disease (PD), the second most common neurodegenerative disorder, oxidative stress is directly linked to the destruction of a specific group of brain cells called dopaminergic neurons, located in a region of the brain known as the substantia nigra pars compacta (SNpc). These are the cells responsible for producing dopamine, a chemical messenger essential for controlling movement.

Ironically, dopamine itself contributes to the oxidative damage in this region through two routes. First, when dopamine is broken down by an enzyme called monoamine oxidase B (MAO-B), hydrogen peroxide is produced as a direct byproduct. Second, dopamine can also undergo spontaneous chemical breakdown on its own, generating highly reactive molecules that latch onto a protein called α-synuclein, causing it to misfold and clump together. These clumps known as Lewy bodies, are the hallmark feature found in the brain cells of Parkinson's patients, and their formation is closely tied to the oxidative environment created by dopamine metabolism(Burbulla et al., 2017). Iron is present at unusually high concentrations in the SNpc, and the combination of elevated iron and H_2O_2 from dopamine metabolism creates particularly favourable conditions for Fenton chemistry and hydroxyl radical generation within dopaminergic neurons.

Studies conducted on brain tissue from deceased Parkinson's patients have consistently shown that a key component of the mitochondria, known as complex I is significantly less active than normal. Since complex I is one of the main sites where superoxide is generated, any reduction in its function disrupts the cell's energy production while simultaneously increasing oxidative stress. These same studies have also found elevated levels of oxidative damage markers, including oxidized glutathione and 4-HNE in the affected brain region. Taken together, these findings confirm that the dopamine-producing neurons lost in Parkinson's disease are subjected to a state of chronic and severe oxidative stress long before they die.(Schapira, 2007).

Further support for the role of mitochondrial dysfunction and oxidative stress in Parkinson's disease comes from studies using environmental toxins. When a chemical called MPTP is administered to animals, it is converted in the brain into a toxic substance that directly blocks complex I, the same mitochondrial component found to be defective in Parkinson's patients. This selectively kills the dopamine-producing neurons in the SNpc and produces movement problems that closely mirror the symptoms of Parkinson's disease in both primates and rodents, making it a widely used experimental model of the disease. Similarly, rotenone, a naturally occurring pesticide that also blocks complex I, produces the same pattern of dopaminergic cell loss, along with α-synuclein clumping and mitochondrial oxidative stress, when administered to animals. Notably, epidemiological studies have found that agricultural workers with prolonged exposure to rotenone have a significantly higher risk of developing Parkinson's disease, providing real-world human evidence that links mitochondrial oxidative damage to the disease(Schapira, 2007).

Neuroinflammation and oxidative stress do not act independently, they fuel each other in a cycle that accelerates brain cell damage. When the brain detects a threat, such as misfolded protein clumps, dying neurons, or invading pathogens, its resident immune cells, known as microglia, become activated. Reactive astrocytes, another type of brain support cell, also join this response. Together, these activated cells release large amounts of free radicals and other reactive molecules as part of their attempt to neutralize the perceived threat. While this inflammatory response is intended to be protective, when it becomes

chronic and excessive, the sheer volume of reactive molecules produced ends up damaging the very neurons these cells are meant to defend(Ginhoux et al., 2010)

2.2 Plant Secondary Metabolites as Natural Antioxidants

The antioxidant capacity of plants resides primarily in their secondary metabolite content, particularly the large and chemically diverse group of phenolic compounds(Dai & Mumper, 2010). Phenolics are biosynthesized via the shikimate and phenylpropanoid pathways and are structurally defined by the presence of one or more hydroxyl groups attached directly to an aromatic ring(Croteau et al., 2000). This structural feature is central to antioxidant function: the hydroxyl group can donate a hydrogen atom, along with its associated electron, to a free radical, converting it into a stable, non-reactive species. The phenoxy radical generated in this process is itself stabilized by resonance delocalization of the unpaired electron across the aromatic ring system, preventing it from readily initiating further radical chain reactions(Rice-Evans et al., 1996).

Flavonoids represent the most studied subclass of plant phenolics, with over 9,000 distinct structures identified to date(Middleton Jr et al., 2000). They share a common C6-C3-C6 skeleton consisting of two benzene rings (A and B) linked via an oxygenated three-carbon bridge forming a central heterocyclic ring (C). Based on the degree of oxidation and pattern of substitution of the C-ring, flavonoids are classified into major subclasses including flavones, flavonols, flavanones, isoflavones, anthocyanidins, and catechins(Manach et al., 2004). The antioxidant mechanisms of flavonoids are multi-dimensional: they act as direct

free radical scavengers through hydrogen atom transfer (HAT) and single electron transfer (SET); chelate redox-active transition metals such as iron and copper, preventing Fenton-type radical generation; inhibit pro-oxidant enzymes including xanthine oxidase and lipoxygenase; and activate Nrf2-ARE signaling, upregulating endogenous antioxidant enzyme expression(Williams et al., 2004).

Terpenoids, while less frequently highlighted in antioxidant studies than phenolics, contribute meaningfully to antioxidant activity — particularly in non-polar extracts(Bakkali et al., 2008). Carotenoids, a prominent class of tetraterpenes, are well-established antioxidants that quench singlet oxygen through energy transfer mechanisms and scavenge peroxyl radicals, providing protection particularly in lipid-rich environments such as cellular membranes(Stahl & Sies, 2003). The total antioxidant capacity of any plant extract reflects the combined, often synergistic activity of multiple compound classes operating through different chemical mechanisms(Crozier et al., 2009). This chemical complexity is precisely why a multi-assay approach, as employed in the present study provides a more informative and scientifically defensible characterization of antioxidant potential than relying on any single test.

2.3 Overview of Antioxidant Assays Employed

A fundamental methodological consideration in antioxidant properties research is that no single in vitro assay is capable of capturing the full complexity of antioxidant activity. Different assays operate through different chemical mechanisms, involve different reactive species, and respond differently to different structural classes of antioxidant

molecules(Prior et al., 2005). The use of a complementary panel of assays is therefore strongly recommended in the current scientific literature, both to improve the reliability and reproducibility of conclusions and to provide mechanistic insights into the nature of the antioxidant activity observed(Huang et al., 2005). Seven assays were selected for this purpose in the present study, as described in the following subsections.

2.3.1 DPPH Radical Scavenging Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, first described by Blois in 1958, is one of the most widely adopted and standardized methods for measuring the hydrogen atom transfer (HAT) and single electron transfer (SET) capacity of antioxidants(Blois, 1958). DPPH is a stable synthetic nitrogen-centred radical with a characteristic deep violet colour and maximum absorbance at approximately 515–517 nm. Upon reaction with an antioxidant that donates a hydrogen atom or electron, the DPPH radical is reduced to the pale yellow, non-radical DPPH-H form, causing a measurable decrease in absorbance proportional to the amount of antioxidant present(Brand-Williams et al., 1995). Results are typically expressed as the IC50 value, the concentration of extract required to reduce the initial DPPH absorbance by 50% with lower IC50 values indicating stronger scavenging activity. Ascorbic acid and/or Trolox (±-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) are used as positive reference antioxidants(Molyneux & others, 2004).

2.3.2 Total Antioxidant Capacity (TAC) — Phosphomolybdenum Method

The Total Antioxidant Capacity assay using the phosphomolybdenum method, as described by Prieto et al., measures the capacity of an extract to reduce Mo(VI) to Mo(V)

under acidic conditions, forming a stable green phospho-Mo(V) complex with maximum absorbance at 695 nm(Prieto et al., 1999). Unlike the DPPH assay, which is conducted in an organic solvent system at ambient temperature, the phosphomolybdenum TAC assay is conducted in an aqueous acidic medium at elevated temperature (90°C) and captures both electron transfer and hydrogen atom transfer antioxidant mechanisms(Umamaheswari & Chatterjee, 2008).

2.3.3 Hydroxyl Radical Scavenging Activity (HRSA)

The hydroxyl radical (•OH) is widely regarded as the most damaging ROS in biological systems, capable of reacting with virtually all classes of biological molecules at near-diffusion-limited rates with no enzymatic mechanism for direct scavenging(Sies et al., 2017). The HRSA assay employs the deoxyribose degradation method developed by Halliwell et al., in which hydroxyl radicals are generated via the Fenton reaction (Fe^{2+} + $H^{2}02$ → Fe^{3+} + •OH + OH^{-}) and allowed to degrade 2-deoxyribose(Halliwell et al., 1987). The degradation products react with thiobarbituric acid (TBA) to produce a pink-coloured chromogen detectable at 532 nm. Antioxidants that compete with deoxyribose for hydroxyl radicals reduce the degree of deoxyribose degradation, and this reduction is quantified as percent scavenging activity(Sakat et al., 2010).

2.3.4 Total Phenolic Content (TPC) — Folin-Ciocalteu Method

The TPC was determined using the Folin-Ciocalteu reagent method, originally described by Singleton and Rossi and subsequently refined for use with diverse plant extracts(Singleton & Rossi Jr, 1965). The Folin-Ciocalteu reagent — a mixture of phosphomolybdic and phosphotungstic acid complexes — is reduced by phenolic

compounds under alkaline conditions (sodium carbonate), producing a blue-coloured molybdenum-tungsten complex with maximum absorbance at approximately 760 nm(Vl, 1999). TPC is quantified from a gallic acid calibration curve and expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g). While TPC does not identify individual phenolic compounds, it provides a reliable and widely accepted estimate of total phenolic richness that is routinely correlated with antioxidant activity data to establish whether phenolics are the primary drivers of observed activity(Naczk & Shahidi, 2006).

2.3.5 Total Flavonoid Content (TFC)

The TFC was measured using the aluminium chloride colorimetric method described by Chang et al(Chang et al., 2002). Aluminium ions (Al^{3+}) form stable chelate complexes with flavonoids possessing a free hydroxyl group adjacent to a carbonyl group (as in flavones and flavonols) or a 3-OH group, producing a characteristic yellow colour with maximum absorbance at approximately 420 nm(Zhishen et al., 1999). Results are expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g). TFC data complement TPC by providing information specifically about the flavonoid sub-fraction, which is pharmacologically significant given the diverse and well-characterized biological activities of flavonoids(Kumar & Pandey, 2013).

2.3.6 Lipid Peroxidation Inhibition

Lipid peroxidation is a critical pathway of oxidative damage with direct relevance to membrane integrity, cardiovascular disease, and neurodegeneration(Gutteridge & Halliwell, 2000). In the present study, lipid peroxidation inhibition was assessed using the thiobarbituric acid reactive substances (TBARS) method, in which peroxidation is

induced in a model lipid substrate by ferric chloride and gallic acid and the extent of peroxidation is quantified by the pink chromogen formed from the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) at 532 nm(Ohkawa et al., 1979). Extracts that inhibit lipid peroxidation reduce MDA production and decrease absorbance; results are expressed as percent inhibition relative to the uninhibited control(Duh et al., 1999).

2.3.7 Ferric Reducing Antioxidant Power (FRAP)

The Ferric Reducing Antioxidant Power (FRAP) assay, originally described by Benzie and Strain, measures the capacity of an extract to reduce ferric tripyridyltriazine complex $[Fe(III)\text{-}(TPTZ)]^{2+}$ to the intensely blue ferrous form $[Fe(II)\text{-}(TPTZ)]^{2+}$ at low pH, with maximum absorbance at 700 nm(Benzie & Strain, 1996). The FRAP value directly reflects the electron-donating (reductive) capacity of the extract and specifically measures antioxidant activity through the single electron transfer (SET) mechanism(Pulido et al., 2000)

Chapter 3: Materials and Method

3.1 Plant Material

Whole plant material of *H.corymbosa* (L.) Lam. was obtained in dried and powdered form from. A total of 500 g of the powdered plant material was procured for the study. Purchasing commercially dried and powdered plant material from established herbal suppliers is an accepted practice in pharmacognostic research when the primary objective is phytochemical and biological screening rather than voucher-specimen-based taxonomic work; however, it is acknowledged as a limitation that formal herbarium authentication of the collected material was not undertaken in the present study(Tiwari et al., 2011).

Prior to extraction, the powder was visually inspected for signs of fungal contamination, insect infestation, or adulteration. The material was stored in a cool, dry location away from direct sunlight in sealed containers until use, to minimize oxidative degradation of secondary metabolites.

3.2 Preparation of Extracts

3.2.1 Primary Methanol Extraction

Extraction was performed by the maceration method. A total of 450 g of the dried powdered plant material was accurately weighed and transferred to a clean, glass container. Approximately 800 ml of analytical-grade methanol was added to the plant powder, and the mixture was allowed to soak at room temperature for 24 hours with occasional manual stirring to facilitate solute diffusion from the plant matrix into the solvent.

After 24 hours, the mixture was filtered to collect the methanolic filtrate. The marc was re-soaked in a fresh volume of methanol and the maceration process was repeated for a second and a third time under the same conditions. This triple maceration approach is recommended to maximize extraction efficiency, as a single extraction cycle typically recovers only a fraction of the total extractable solutes(Sultana et al., 2009). The filtrates from all three cycles were pooled, yielding approximately 500 ml of combined methanolic filtrate.

The pooled filtrate was concentrated under reduced pressure using a rotary evaporator at a water bath temperature not exceeding 45°C, to prevent thermal degradation of heat-sensitive phenolic compounds. The concentrated extract was subsequently subjected to freeze-drying (lyophilization) to remove residual solvent and obtain a dry, stable solid extract. The weight of the concentrate prior to freeze-drying was recorded as 30.93 g. After freeze-drying, the final weight of the crude methanol extract was 23.2 g. Of the total 23.2 g of crude methanol extract, 2.0 g was set aside for direct biological testing as the crude methanol extract, and the remainder (21.2 g) was used for successive liquid-liquid fractionation as described in the following sections.

3.2.2 Liquid-Liquid Fractionation: n-Hexane Fraction

Liquid-liquid partitioning was employed to fractionate the crude methanol extract into chemically distinct fractions based on the polarity of their constituent compounds. This method is a standard approach in phytochemical research for separating compound classes prior to biological screening or isolation work(Sarker et al., 2006).

The crude methanol extract (21.2 g, excluding the 2 g aliquot reserved for direct testing) was dissolved in 35 ml of methanol. To this, 200 ml of distilled water was added and the mixture was transferred to a 500 ml separating funnel. n-Hexane (100 ml) was added to the funnel, which was then stoppered, inverted, and vigorously shaken. The funnel was then secured on a retort stand and allowed to stand undisturbed for 24 hours to permit complete phase separation.

After 24 hours, two distinct layers were visible: a lower aqueous-methanol layer and an upper n-hexane layer. The n-hexane (upper) layer was carefully drained off and collected. The aqueous-methanol layer was retained in the separating funnel for subsequent fractionation with chloroform. The hexane partitioning process was repeated twice more with fresh 100 ml aliquots of n-hexane, following the same procedure.The three n-hexane fractions were pooled (approximately 200 ml total) and concentrated by rotary evaporation followed by freeze-drying, yielding 3.6 g of dry n-hexane extract.

3.2.3 Liquid-Liquid Fractionation: Chloroform Fraction

Following complete removal of the n-hexane layer, the residual aqueous-methanol layer retained in the separating funnel was used directly for chloroform fractionation. Chloroform (100 ml) was added to the funnel, which was again stoppered, vigorously shaken and allowed to stand for 24-48hours to achieve complete phase separation.

The lower chloroform layer (chloroform is denser than water, with a density of approximately 1.49 g/ml, and therefore settles to the bottom of the separating funnel) was carefully drained off and collected.

The aqueous layer was retained and the process was repeated twice more with fresh 100 ml portions of chloroform. The three chloroform fractions were combined (approximately 300 ml total) and subjected to rotary evaporation, followed by freeze-drying. The final dry chloroform extract weighed 17.6 g.

3.2.4 Summary of Extract Yields

Table 1 below summarizes the extraction yields obtained from the plant material.

Extract/Fraction	Solvent	Total Plant material	Dry extract weight of fractions
Crude/Methanol extract	Methanol	450g	23.3g
n-Hexane fraction	n-Hexane		3.6g
Chloroform fraction	Chloroform		17.6

Table 1: Summary of extract yields

3.3 Chemicals and Reagents

All chemicals and reagents used in the study were of analytical or reagent grade unless otherwise specified. Methanol, chloroform, n-hexane, and distilled water used for extraction were of high-purity grade. Gallic acid (reagent grade) was used as a standard for the TPC assay. Quercetin (analytical grade) was used as a standard for the TFC assay. Ascorbic acid (analytical grade) was used as a positive control for the total antioxidant capacity, and hydroxyl radical scavenging assays. BHT, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), potassium acetate, ammonium molybdate, sodium phosphate (Na_3PO_4), concentrated sulfuric acid (H_2SO_4, 98%), DPPH (1,1-diphenyl-2-picrylhydrazyl), 2-deoxy-D-ribose, thiobarbituric acid (TBA), EDTA, hydrogen peroxide (H_2O_2),

ferric chloride ($FeCl_3$), trichloroacetic acid (TCA), catechin, and potassium phosphate buffer salts (KH_2PO_4 and K_2HPO_4) were all sourced from reputable chemical suppliers. All aqueous solutions were prepared in freshly boiled and cooled double-distilled water.

3.4 Phytochemical Screening and Antioxidant properties

Preliminary qualitative phytochemical screening was performed on the three extracts (n-hexane, chloroform, and methanol) to determine the general classes of secondary metabolites present. And then antioxidant assays were performed.

3.5 Determination of Total Phenolic Content (TPC)

3.5.1 Principle

The TPC of the extracts was determined using the Folin-Ciocalteu reagent (FCR) method, originally described by Singleton and Rossi (1965) and subsequently adapted for use with plant extracts(Singleton & Rossi Jr, 1965). The Folin-Ciocalteu reagent is a complex mixture of heteropolyphosphotungstate-molybdate ions that functions as an electron transfer reagent under alkaline conditions. Phenolic compounds, acting as reductants, donate electrons to the Mo(VI)-containing complex, reducing it to Mo(V) through a series of reversible one- and two-electron transfer reactions. The reduced molybdenum complex has a characteristic blue color attributed to species of the form $(PMoW_{11}O_{40})^{4-}$, with maximum absorbance at approximately 760 nm. Results are expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g).

3.5.2 Reagents

1. Folin-Ciocalteu reagent (diluted 10-fold in distilled water at time of use)
2. Sodium carbonate solution (Na_2CO_3, 7.5% w/v in distilled water)

3. Gallic acid standard stock solution (1 mg/ml in methanol; diluted to prepare calibration curve)
4. Methanol (analytical grade)
5. Distilled water

3.5.3 Preparation of Gallic Acid Calibration Curve

A stock solution of gallic acid (1.0 mg/ml) was prepared by dissolving an accurately weighed quantity of gallic acid in methanol. Working standards of concentrations 6.25, 12.5, 25, 50 and 100 µg/ml were prepared by serial dilution of the stock solution with methanol. Each standard concentration was assayed in triplicate alongside the samples, and a linear calibration curve was constructed by plotting absorbance at 760 nm against gallic acid concentration (µg/ml).

3.5.4 Experimental Procedure

1. 0.5 ml of plant extract solution (prepared at an appropriate concentration in methanol) or gallic acid standard solution was pipetted into a clean test tube.
2. 2.5 ml of Folin-Ciocalteu reagent (diluted 10-fold with distilled water) was added and mixed thoroughly2.5 ml of sodium carbonate solution (7.5% w/v) was added immediately after, and the mixture was mixed again.
3. The test tubes were incubated at 25°C for 20 minutes in the dark to allow complete colour development.
4. Absorbance was measured at 760 nm against a blank using a UV-visible spectrophotometer.
5. The blank consisted of all reagents with no extract or standard, processed identically.
6. All measurements were performed in triplicate (n = 3) and results expressed as mean absorbance.

3.5.5 Calculation

The TPC was calculated using the formula:

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

Where C = total phenolic content of the plant extract (mg GAE/g dry extract); c = concentration of gallic acid established from the calibration curve (mg/ml); V = volume of extract solution used (ml); and m = weight of dry plant extract (g).

3.6 Determination of Total Flavonoid Content (TFC)

3.6.1 Principle

The TFC was determined by the aluminium chloride colorimetric method. Aluminium ions (Al^{3+}) form stable chelate complexes with flavonoids that possess adjacent free hydroxyl groups (as found in the 3- and 4-positions of flavonols, or the 4-keto and 5-hydroxyl positions of flavones). This complexation produces a bathochromic shift, resulting in yellow-colored products with maximum absorbance at approximately 415–420 nm. The method specifically targets flavonoids over other phenolic compounds because most simple phenolic acids and tannins do not form stable aluminium chelates under the assay conditions(Zhishen et al., 1999). Results are quantified against a quercetin calibration curve and expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g).

3.6.2 Reagents

1. Aluminium chloride solution ($AlCl_3$ 10g in 100ml distilled water)
2. Potassium acetate solution (1 M in distilled water)

3. Quercetin standard stock solution
4. Methanol (analytical grade)
5. Distilled water

3.6.3 Experimental Procedure

1. 1.0 ml of plant extract solution or quercetin standard solution (prepared at appropriate concentrations in methanol) was transferred to a clean test tube.

2. 3.0 ml of methanol was added and mixed.

3. 200 µl of aluminium chloride solution was added and then 200 µl of potassium acetate solution (1 M) was added.

4. 5.6 ml of distilled water was added and the mixture was thoroughly mixed. The total reaction volume was 10 ml.

5. The tubes were incubated at room temperature (25 ± 2°C) for 30 minutes to allow complete complex formation.

6. Absorbance was measured at 420 nm using a UV-visible spectrophotometer against a blank.

7. The blank consisted of all reagents with distilled water substituted for the $AlCl_3$ solution, to account for any non-specific colour from the extract itself.

8. All measurements were performed in triplicate (n = 3). TFC was calculated from the quercetin calibration curve and expressed as mg QE/g dry extract.

3.7 Determination of Total Antioxidant Capacity (TAC)

3.7.1 Principle

The total antioxidant capacity was determined by the phosphomolybdenum method. This method is based on the reduction of Mo(VI) to Mo(V) by antioxidant compounds in an acidic environment, with the subsequent formation of a green-colored phosphate/Mo(V) complex. The reduction reaction is driven by the electron-donating capacity of antioxidants present in the extract, including ascorbic acid, phenolic compounds, α-tocopherol, and carotenoids. The green phospho-Mo(V) complex exhibits maximum absorbance at 695 nm, and the absorbance is proportional to the total electron-donating (antioxidant) capacity of the extract.

3.7.2 Reagents

1. Phosphomolybdenum reagent solution (0.6 M H_2SO_4 + 28 mM Na_3PO_4 + 4 mM ammonium molybdate)
2. Ascorbic acid standard stock solution
3. Concentrated sulfuric acid (H_2SO_4, 98%)
4. Sodium phosphate (Na_3PO_4)
5. Ammonium molybdate

3.7.3 Experimental Procedure

1. 1 ml of plant extract solution (dissolved in the appropriate solvent at suitable concentrations) or ascorbic acid standard of concentrations 3.25, 6.25, 12.5, 25, 50 and 100µg/ml was transferred to test tubes
2. 3 ml of the phosphomolybdenum reagent solution was added to each tube and the contents were mixed thoroughly.

3. The tubes were incubated in a water bath at 90°C for 90 minutes. The elevated temperature facilitates the reduction of Mo(VI) to Mo(V) and colour development.
4. After incubation, the tubes were removed from the water bath and allowed to cool to room temperature (approximately 25°C) before absorbance measurement.
5. Absorbance was measured at 695 nm against a blank using a UV-visible spectrophotometer.
6. The blank consisted of 3 only methanol. The positive control consisted of 3ml reagent mixture and 1 ml methanol.

3.8 DPPH Free Radical Scavenging Activity

3.8.1 Principle

The DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was performed on all three extracts. DPPH is a stable nitrogen-centred free radical with a characteristic deep violet colour in methanolic solution ($\lambda max \approx 517$ nm). When DPPH encounters an antioxidant molecule capable of donating a hydrogen atom or electron, the radical is reduced to the non-radical form (DPPH-H), resulting in a progressive colour change from violet to yellow and a corresponding decrease in absorbance at 517 nm. The degree of absorbance reduction is directly proportional to the radical scavenging capacity of the antioxidant. Ascorbic acid was used as standard.

3.8.2 Reagents

1. DPPH solution (4mg in 100ml methanol)

2. Plant extract solutions (prepared at 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml in methanol)
3. Ascorbic acid
4. Methanol (analytical grade)

3.8.3 Experimental Procedure

1. 2ml o plant extract or ascorbic acid standard solution (at various concentrations) in methanol was transferred to a clean test tube wrapped in aluminium foil to protect from light.
2. 2ml of DPPH solution in methanol was added to each tube.
3. The tubes were incubated at room temperature (25 ± 2°C) for 30 minutes in a dark place. Exclusion of light is essential because DPPH is photosensitive and undergoes non-radical-mediated degradation upon light exposure.
4. Absorbance was measured at 517 nm against a methanol blank using a UV-visible spectrophotometer.
5. The blank consisted of all reagents except the plant extract (i.e., methanol in place of extract), processed identically.
6. The percentage inhibition of DPPH radical scavenging activity was calculated using the equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 is the absorbance of the control (DPPH solution without extract) and A_1 is the absorbance of the test sample (DPPH + extract).

3.9 Hydroxyl Radical Scavenging Activity (HRSA)

3.9.1 Principle

The hydroxyl radical scavenging assay was performed based on the deoxyribose degradation method. In this assay, hydroxyl radicals are generated in situ via the Fenton reaction, in which ferric iron (Fe^{3+}) is first reduced to ferrous iron (Fe^{2+}) by ascorbic acid, and Fe^{2+} subsequently reacts with hydrogen peroxide ($H2O2$) to produce hydroxyl radicals: $Fe^{2+} + H2O2 \rightarrow Fe^{3+} + \bullet OH + OH^-$(Liochev, 2013). These hydroxyl radicals degrade 2-deoxy-2-ribose into a mixture of fragments that, upon heating with thiobarbituric acid (TBA) under acidic conditions, condense to form a pink-coloured chromogen (TBA-reactive species) with maximum absorbance at 532 nm. Antioxidants that compete with deoxyribose for the available hydroxyl radicals, either by direct radical scavenging or through chelation of the iron ions necessary for radical generation reduce the extent of deoxyribose degradation and hence the absorbance signal. BHT was used as the referemce.

3.9.2 Reagents

1. 2-Deoxy-2-ribose
2. Potassium phosphate buffer
3. $FeCl_3$ solution
4. EDTA solution
5. Hydrogen peroxide, H_2O_2
6. Ascorbic acid
7. Trichloroacetic acid (TCA)
8. Thiobarbituric acid (TBA)
9. Butylated hydroxytoluene (BHT)
10. Methanol

3.9.3 Experimental Procedure

1. Buffer preparation: 100 ml of potassium buffer was prepared and pH adjusted to 7.4.
2. All reagents except 2-deoxy-2ribose were taken in a beaker (Beaker R1) and dissolved with the freshly prepared 100ml buffer solution.
3. 2.8g of TCA was dissolved in 100ml of distilled water, and 1g of TBA was dissolved in 100ml distilled water to prepare solutions.
4. Stock solution of Sample extract and standard were prepared.
5. **Blank preparation:**
    - **a.** Blank-1: 1ml of solution from Beaker R1
    - **b.** Blank-2: 1ml solution from Beaker R1 and standard
    - **c.** Blank-3: 1ml solution from Beaker R1 and sample
    - **d.** Blank-4: Add 2-deoxy-2-ribose in Beaker R1, it is now labelled as Beaker R2. Take 1 ml from Beaker R2.
6. Serial dilution of sample and standard solution were prepared to make following concentrations using stock solution and Beaker R2 reagent- 1.56, 3.125, 6.25, 12.5 and 25µg/ml.
7. The mixture was incubated at 37°C in hot water bath for 1 hour.
8. After it was cooled, 2ml of TCA and 2ml of TBA solution was added to each test tubes and again incubated at 90°C for 15 minutes.
9. Absorbance was measured at 532nm.

10. The percentage hydroxyl radical scavenging activity was calculated using the equation: $\% \ Inhibition = [(A_0 - A_1) / A_0] \times 100$

Where A*0* is the absorbance of the control (no extract) and A*1* is the absorbance in the presence of the extract or standard.

3.10 Lipid Peroxidation Inhibition Assay

3.10.1 Principle

Lipid peroxidation is the oxidative breakdown of polyunsaturated fatty acids (PUFAs) in cell membranes, and is one of the most damaging consequences of uncontrolled free radical activity in biological systems. In this assay, lipid peroxidation is induced in mouse brain tissue homogenate using ferric chloride ($FeCl_3$) and Gallic acid, which together generate hydroxyl radicals through the Fenton reaction and initiate the peroxidation of membrane lipids(Gutteridge & Halliwell, 2000). The extent of peroxidation is measured by the thiobarbituric acid reactive substances (TBARS) method, in which malondialdehyde (MDA), a major end product of lipid peroxidation, reacts with TBA under acidic, high-temperature conditions to produce a pink-coloured chromogen that absorbs light at 532 nm(Duh et al., 1999)

3.10.2 Reagents

1. Rat brain homogenate
2. $FeCl_3$ solution (3.22mg in 100ml distilled water)
3. Gallic acid solution (Standard)
4. Trichloroacetic acid (TCA)

5. Thiobarbituric acid (TBA)
6. Potassiul Chloride (KCl)
7. Hydrochloric acid (HCl)
8. Butylated hydroxytoluene (BHT)
9. Plant extract solutions (at various concentrations in appropriate solvent)

3.10.3 Experimental Procedure

1. Prepare 100ml of phosphate buffer and 100 ml of KCl solution and keep it in the freezer.
2. 100 ml solution containing HCl, TCA, TBA and BHT was prepared and kept in a freezer
3. Take out rat brain and make a mashed mixture with buffer and KCl in a 1:4.5:4.5 ratio. For 1g rat brain, 4.5ml buffer and 4.5ml KCl solution.
4. This ice cold mixture is then taken in a falcone tube and centrifuged at 10,000 rpm for 15 minutes.
5. Supernatant was collected in another falcone tube.
6. Then stock solutions of sample and standard were prepared and serial dilution performed to get the following concentrations 6.25, 12.5, 25, 50, 100 and 200µg/ml.
7. In the falcone tubes containing the above concentrations, 1ml KCl, 0.5ml of rat brain homogenate supernatant and 100µL of $FeCl_3$ added and incubated at 37°C for 30 minutes.

8. Falcone tubes were cooled to room temperature. Then 2ml of ice cold solution containing TCA, TBA, HCl and BHT were added in each tubes. The mixture was heated again for 1 hour at 80℃
9. After bringing it to room temperature, it was centrifuged at 5000rpm for 10 minutes and supernatant collected in test tubes.
10. Absorbance was measured at 532nm and %I was calculated using the following formula.

$$\% \ Inhibition = [(A_0 - A_1) / A_0] \times 100$$

3.11 Ferric Reducing Antioxidant Power (FRAP) Assay

3.11.1 Principle

In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, Fe2+ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

3.11.2 Reagents

1. Potassium ferricyanide [K3Fe(CN)6]
2. $FeCl_3$ solution
3. Potassium phosphate buffer
4. TCA

5. Ascorbic acid standard solutions

6. Plant extract solutions (in appropriate solvent)

3.11.3 Experimental Procedure

1. 100ml of potassium phosphate buffer, 100ml of TCA, 100 ml of FeCl3 and 100ml of Potassium ferricyanide solutions were prepared and set aside. All reagents were dissolved in distilled water to get the 100 ml solutions.
2. Stock solution of sample and standard was prepared and serial dilution was performed to get concentrations 6.25, 12.5, 25, 50 and 100µg/ml.
3. After pipetting the concentrations, 2.5ml of potassium ferricyanide, 2.5ml of buffer was added to each falcone tube and incubated at 50°C for 20 minutes.
4. Then 2.5 ml of TCA was added and the falcone tubes were centrifuged at 3300rpm for 10 minutes. 2.5ml of supernatant from each tube was collected.
5. 2.5 ml od distilled water and 0.5ml of FeCl3 was added next.
6. Absorbance was measured at 700nm against blank containing only methanol and control containing all reagents except standard and sample.

Chapter 4: Result

4.1 Total Phenolic Content (TPC)

The TPC of the three extracts of *H.corymbosa* was determined using the Folin-Ciocalteu (FC) colorimetric method with gallic acid as the reference standard. A five-point calibration curve was constructed from gallic acid standard solutions (6.25–100 µg/ml) measured in triplicate at 760 nm. The calibration equation was $y = 0.0078x + 0.0269$ ($R^2 = 0.9827$), confirming excellent linearity. Extract TPC values were calculated using the formula $C = (c \times V) / m$, where c is the gallic acid equivalent concentration (mg/ml) from the calibration curve, V is the sample volume (0.5 ml), and m is the dry extract weight (0.001 g). Each extract was measured at 1000 µg/ml in triplicate (n = 3). Results are expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g,).

4.1.1: Standard absorbance and standard curve

Concentration(µg/ml)	Absorbance	Average
6.25	0.037	0.033
	0.0311	
	0.0329	
12.5	0.1114	0.109
	0.104	
	0.1126	
25	0.2607	0.267
	0.2882	
	0.2526	
50	0.4462	0.455
	0.4638	
	0.4559	
100	0.7524	0.780
	0.7819	
	0.8076	

Table 2: Gallic acid calibration curve absorbance values (760 nm) used for TPC calculation

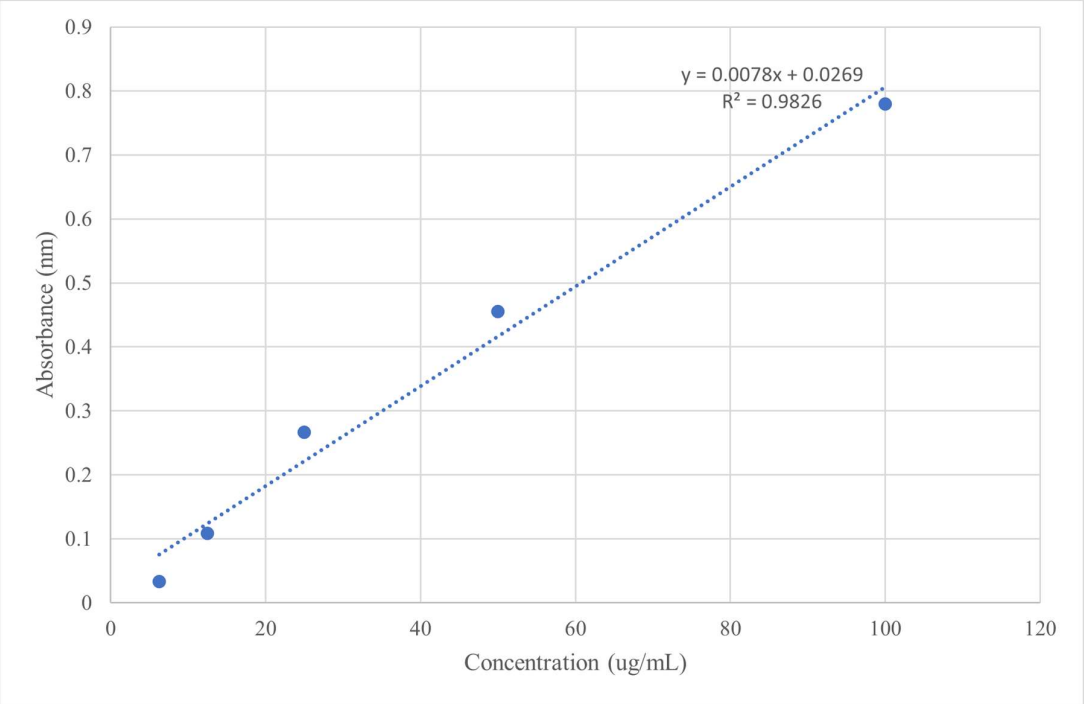

Figure 1: Standard curve of Gallic acid

4.1.2: Crude absorbance and mean TPC

Concentration (ug/ml)	m=weight of dried mass (g)	Absorbance	$x = \frac{y - 0.0269}{0.0078}$	C (mg/ml)	TPC= (CxV)/m	Mean TPC mg GAE/g
1000	0.001	0.671	82.57	0.0825	41.25	42.01
1000	0.001	0.6983	86.07	0.086	43	
1000	0.001	0.6791	83.61	0.0836	41.8	

Table 3: Mean TPC of crude methanol extract

4.1.3: Chloroform extract absorbance and mean TPC

Concentration (ug/ml)	m=weight of dried mass (g)	Absorbance	$x = \frac{y - 0.0269}{0.0078}$	C (mg/ml)	TPC= (CxV)/m	Mean TPC mg GAE/g
1000	0.001	0.7801	96.56	0.0965	48.25	47.75
1000	0.001	0.7745	95.84	0.0958	47.9	
1000	0.001	0.7618	94.21	0.0942	47.1	

Table 4: Mean TPC of chloroform extract

4.1.4: n-Hexane extract absorbance and mean TPC

Concentration (ug/ml)	m=weight of dried mass (g)	Absorbance	$x = \frac{y - 0.0269}{0.0078}$	C (mg/ml)	TPC= (CxV)/m	Mean TPC mg GAE/g
1000	0.001	0.363	43.08	0.04308	21.54	21.29
1000	0.001	0.3581	42.46	0.04246	21.23	
1000	0.001	0.3562	42.21	0.04221	21.105	

Table 5: Mean TPC of n-Hexane extract

4.1.5: Comparison between three extracts

The chloroform extract contained the highest total phenolic content (47.77 mg GAE/g), followed by the crude methanol extract (42.04 mg GAE/g) and the n-hexane extract (21.29 mg GAE/g). The individual TPC values calculated from each replicate absorbance reading were consistent within each extract group, validating the reliability of the assay performance across all three fractions

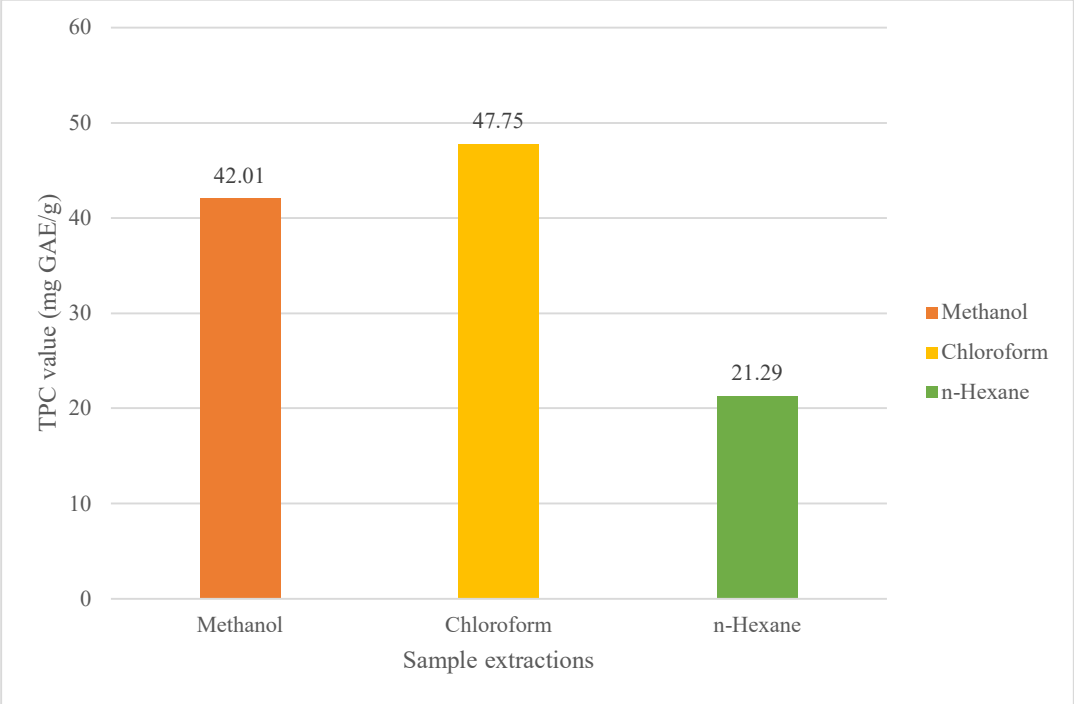

Figure 2: Comparison of mean TPC of methanol, chloroform and n-hexane extracts

4.2 Total flavonoid content (TFC)

The TFC was measured using the aluminium chloride ($AlCl_3$) colorimetric method, with absorbance recorded at 420 nm and quercetin used as the calibration standard. A five-point standard curve was constructed from quercetin solutions (6.25–100 µg/ml) measured in triplicate, yielding the calibration equation $y = 0.0066x + 0.0494$ ($R^2 = 0.9761$). TFC values were calculated using the same formula as TPC: $C = (c \times V) / m$ ($V = 0.5$ ml, $m = 0.001$ g), with c derived from the quercetin calibration curve. Results are expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g). Each extract was tested in triplicate at 1000 µg/ml.

4.2.1: Standard absorbance and standard curve

Concentration(µg/ml)	Absorbance	Average
6.25	0.052	0.0488
	0.0465	
	0.0481	
12.5	0.1375	0.1217
	0.1104	
	0.1172	
25	0.2461	0.2421
	0.2386	
	0.2417	
50	0.4481	0.4281
	0.4193	
	0.417	
100	0.6827	0.6755
	0.6725	
	0.6713	

Table 6: Qquercetin absorbance to make standard curve for the calculation of TFC

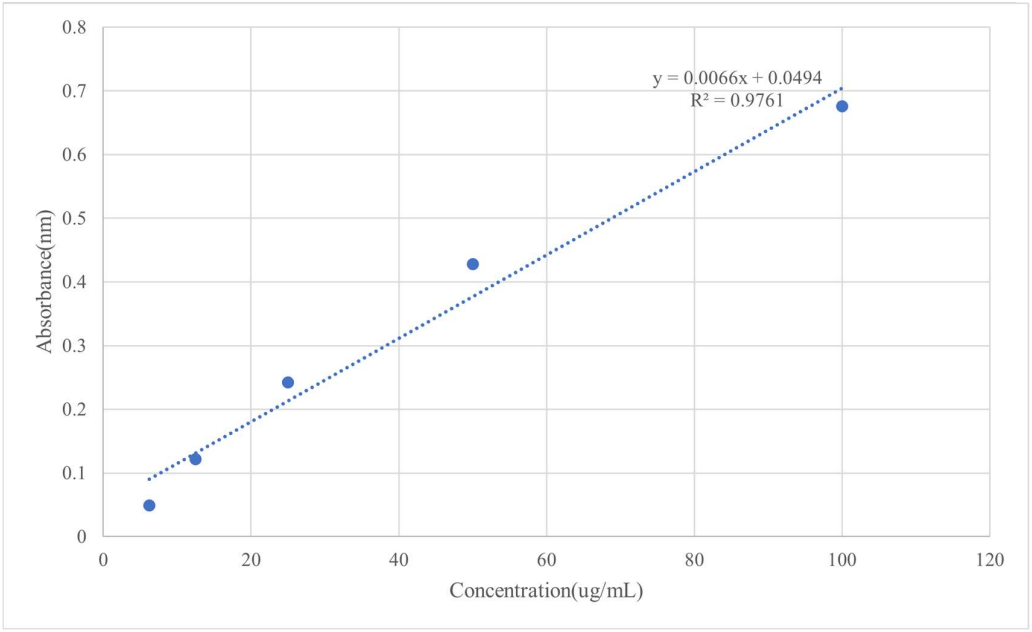

Figure 3: Standard curve of quercetin

4.2.2: Crude absorbance and mean TFC

Concentration (ug/ml)	m=weight of dried mass (g)	Absorbance	$x = \frac{y - 0.0494}{0.0066}$	C (mg/ml)	TFC= (CxV)/m	Mean TFC mg GAE/g
1000	0.001	0.415	55.39	0.055	27.5	28.83
1000	0.001	0.4473	60.28	0.060	30	
1000	0.001	0.4358	58.54	0.058	29	

Table 7: Methanol extract absorbance and mean TFC

4.2.3: Chloroform absorbance and mean TFC

Concentration (ug/ml)	m=weight of dried mass (g)	Absorbance	$x = \frac{y - 0.0494}{0.0066}$	C (mg/ml)	TFC= (CxV)/m	Mean TFC mg GAE/g
1000	0.001	0.4785	65.01	0.065	32.5	32.85
1000	0.001	0.4932	67.24	0.0672	33.6	
1000	0.001	0.4779	64.92	0.0649	32.45	

Table 8: Chloroform extract absorbance and mean TFC

4.2.4: n-Hexane absorbance and mean TFC

Concentration (ug/ml)	m=weight of dried mass (g)	Absorbance	$x = \frac{y - 0.0494}{0.0066}$	C (mg/ml)	TFC= (CxV)/m	Mean TFC mg GAE/g
1000	0.001	0.2421	29.19	0.0291	14.55	13.9
1000	0.001	0.229	27.21	0.0272	13.6	
1000	0.001	0.2287	27.16	0.0271	13.55	

Table 9: n-Hexane extraction absorbance and mean TFC

4.2.5: Comparison

The chloroform extract recorded the highest TFC (32.85 mg QE/g), followed by the crude methanol extract (28.83 mg QE/g) and the n-hexane extract (13.9 mg QE/g). The rank order of TFC was identical to that observed for TPC.

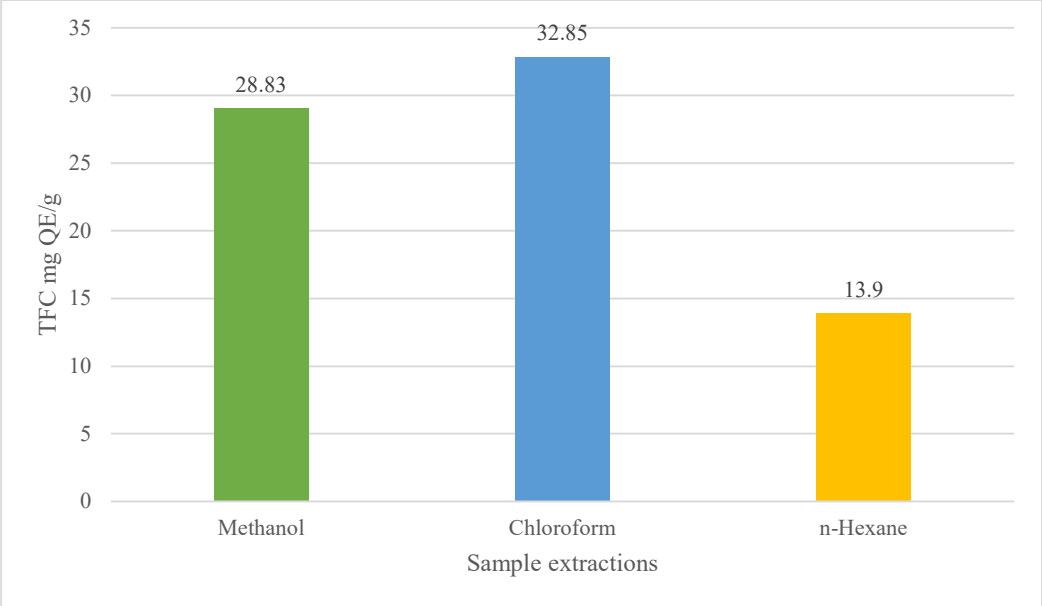

Figure 4: Comparison between TFC values of methanol, chloroform and n-hexane extract

4.3 DPPH free radical scavenging assay

The DPPH free radical scavenging activity of the three extracts of *H.corymbosa* was measured across six concentrations (3.125–100 µg/ml), with Ascorbic acid as the positive control standard. The control absorbance (DPPH solution without extract) was recorded as 0.553. Percentage inhibition (% I) was calculated for each concentration using the formula $\% I = [(A_0 - A_1) / A_0] \times 100$, where A_0 is the control absorbance and A_1 is the sample absorbance.

4.3.1 Standard absorbance and %Scavenging

Concentration(µg/ml)	Absorbance	Average	%Scavenging
3.125	0.4614	0.4791	13.3%
	0.4725		
	0.5035		
6.25	0.4297	0.4493	18.7%
	0.4767		
	0.4417		
12.5	0.3266	0.3311	40.1%
	0.3132		
	0.3537		
25	0.2216	0.2393	56.7%
	0.2565		
	0.2398		
50	0.1116	0.1107	79.9%
	0.1078		
	0.1128		
100	0.0516	0.0581	89.4%
	0.0621		
	0.0608		

Table 10: Standard (ascorbic acid) absorbance and %I value for DPPH assay

4.3.2: Methanol extract absorbance and %Scavenging

Concentration(µg/ml)	Absorbance	Average	%Scavenging
3.125	0.3037	0.3373	39%
	0.3693		
	0.339		
6.25	0.3167	0.3216	41.8%
	0.3213		
	0.3269		
12.5	0.3135	0.3016	45.4%
	0.2946		
	0.2967		
25	0.2856	0.2912	47.3%
	0.2961		
	0.2919		
50	0.2722	0.2676	51.6%
	0.2599		
	0.2707		
100	0.2539	0.2362	57.2%
	0.2314		
	0.2233		

Table 11: Methanol extract DPPH assay result

4.3.3: Chloroform extract absorbance and %Scavenging

Concentration(µg/ml)	Absorbance	Average	%Scavenging
3.125	0.356	0.3859	30.2%
	0.405		
	0.3968		
6.25	0.3742	0.3582	35.22%
	0.3554		
	0.345		
12.5	0.3365	0.333	39.7%
	0.3479		
	0.3146		
25	0.3065	0.3133	43.3%
	0.3192		
	0.3142		
50	0.2691	0.2732	50.5%
	0.2746		
	0.2759		
100	0.2004	0.2081	62.4%
	0.2125		
	0.2116		

Table 12: Chloroform extract DPPH assay result

4.3.4: n-Hexane extract absorbance and %Scavenging

Concentration(µg/ml)	Absorbance	Average	%Scavenging
3.125	0.4566	0.463	16.2%
	0.4432		
	0.4892		
6.25	0.4435	0.4498	18.6%
	0.4377		
	0.4682		
12.5	0.3941	0.4023	27.2%
	0.4203		
	0.3926		
25	0.3818	0.3776	31.7%
	0.3844		
	0.3667		
50	0.3658	0.3720	32.7%
	0.3784		
	0.372		
100	0.356	0.353	36.16%
	0.3578		
	0.3453		

Table 13: n-Hexane extract DPPH assay result

4.3.5: Comparison

All three extracts displayed a concentration-dependent reduction in DPPH absorbance, confirming genuine free radical scavenging activity. Ascorbic acid achieved the strongest scavenging with %I of 89.4% at 100ug/ml. Among plant extracts, the crude Chloroform extract showed the highest DPPH activity (%I at 100µg/ml= 62.4%), closely followed by

the methanol extract (%I at 100µg/ml= 57.2%). The n-hexane extract having only 36.16% of %I at 100ug/ml. Notably, the methanol extract showed relatively high scavenging activity even at the lowest tested concentration (39.0 ± 5.9% at 3.125 µg/ml), with activity increasing only gradually thereafter.

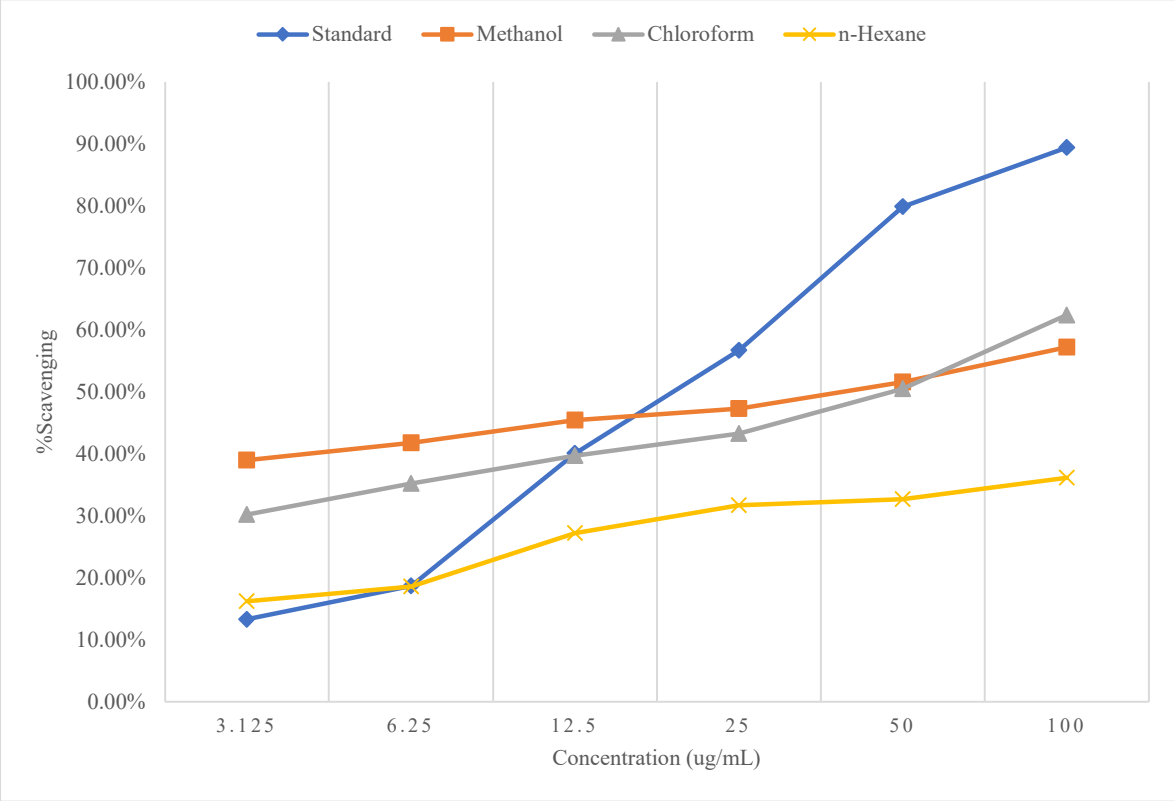

Figure 5: DPPH assay result of Standard, methanol, chloroform and n-Hexane extract

4.4 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was evaluated using the deoxyribose degradation method across five concentrations (1.56–25 µg/ml), with BHT as the positive reference standard. The positive control absorbance (A_0) was calculated as 0.618 (Blank 4 − Blank 1 = 0.650 − 0.032), representing maximal deoxyribose degradation in the absence of any scavenger. Individual extract-specific blank corrections (Blank 3) were

applied to each sample to account for background absorbance contributed by the extracts themselves in the reaction system. Percentage inhibition was calculated as % I = $[(A_0 - A_1) / A_0] \times 100$, where A_1 is the blank-corrected sample absorbance.

Blank 1: Mixture where 2-deoxy 2-ribose is absent- 0.032

Blank 2: Mixture where 2-deoxy 2-ribose is absent but standard is present- 0.053

Blank 3-Crude: Mixture where 2-deoxy 2-ribose is absent but crude is present- 0.055

Blank 3-Chloroform: Mixture where 2-deoxy 2-ribose is absent but chloroform extract is present- 0.042

Blank 3-N-hexane: Mixture where 2-deoxy 2-ribose is absent but n-hexane extract is present- 0.028

Blank 4: Mixture where 2-deoxy 2-ribose is present- 0.650

4.4.1: Result of Standard BHT

Concentration ($\mu g/ml$)	Absorbance	Blank 2 subtraction	Average(A_1)	%Scavenging
1.56	0.6391	0.5861	0.577	6.63%
	0.6324	0.5794		
	0.6192	0.5662		
3.125	0.5828	0.5358	0.5328	13.78%
	0.57	0.524		
	0.5847	0.5387		
6.25	0.5510	0.505	0.5025	18.68%
	0.5334	0.4874		
	0.5611	0.5151		
12.5	0.5182	0.4722	0.4579	25.9%
	0.4994	0.4534		
	0.4942	0.4482		
25	0.4568	0.4108	0.404	34.62%
	0.4595	0.4135		
	0.4337	0.3877		

Table 14: Ascorbic acid result for HRSA

4.4.2: Methanol extract result

Concentration ($\mu g/ml$)	Absorbance	Blank 3-crude subtraction	Average(A_1)	%Scavenging
1.56	0.6691	0.6141	0.5945	3.8%
	0.628	0.573		
	0.6514	0.5964		
3.125	0.6012	0.5462	0.5443	11.95%
	0.5937	0.5387		
	0.6032	0.5482		
6.25	0.5673	0.5123	0.51	17.47%
	0.5569	0.5019		
	0.571	0.516		
12.5	0.5139	0.4589	0.4711	23.77%
	0.5382	0.4832		
	0.5264	0.4714		
25	0.4972	0.4422	0.4342	29.74%
	0.4823	0.4273		
	0.4881	0.4331		

Table 15: Methanol extract result for HRSA assay

4.4.3: Chloroform extract result

Concentration ($\mu g/ml$)	Absorbance	Blank 3-crude subtraction	Average(A_1)	%Scavenging
1.56	0.6363	0.5943	0.5895	4.6%
	0.6249	0.5829		
	0.6334	0.5914		
3.125	0.6102	0.5682	0.5608	9.25%
	0.5937	0.5517		
	0.6047	0.5627		
6.25	0.5613	0.5193	0.5122	17.11%
	0.5568	0.5148		
	0.5445	0.5025		
12.5	0.499	0.457	0.4686	24.17%
	0.5182	0.4762		
	0.5148	0.4728		
25	0.4649	0.4239	0.4266	30.9%
	0.4627	0.4207		
	0.4773	0.4353		

Table 16: Chloroform extract resulf for HRSA

4.4.4: n-Hexane extract result

Concentration ($\mu g/ml$)	Absorbance	Blank 3-crude subtraction	Average(A_1)	%Scavenging
1.56	0.636	0.608	0.6124	0.9%
	0.6414	0.6134		
	0.6438	0.6158		
3.125	0.6261	0.5981	0.5923	4.15%
	0.6242	0.5962		
	0.6107	0.5827		
6.25	0.5863	0.5583	0.5647	8.62%
	0.601	0.573		
	0.5908	0.5628		
12.5	0.5707	0.5427	0.5417	12.34%
	0.5642	0.5362		
	0.5742	0.5462		
25	0.5261	0.4981	0.4971	19.56%
	0.5253	0.4973		
	0.5241	0.4961		

Table 17: n-Hexane extract result for HRSA

4.4.5: Comparison

None of the three extracts nor the BHT standard achieved 50% hydroxyl radical inhibition within the tested concentration range of 1.56–25 µg/ml. At the highest tested concentration amongst samples (25 µg/ml), the chloroform extract showed the greatest scavenging activity (30.9%), marginally close is the methanol extract (29.74%). The n-hexane extract

showed substantially weaker activity at all concentrations (maximum 19.56% at 25 µg/ml). BHT showed 34.62% inhibition at 25ug/ml concentration. All three extracts showed a clear and reproducible linear increase in % inhibition with rising concentration, confirming genuine dose-dependent scavenging behaviour.

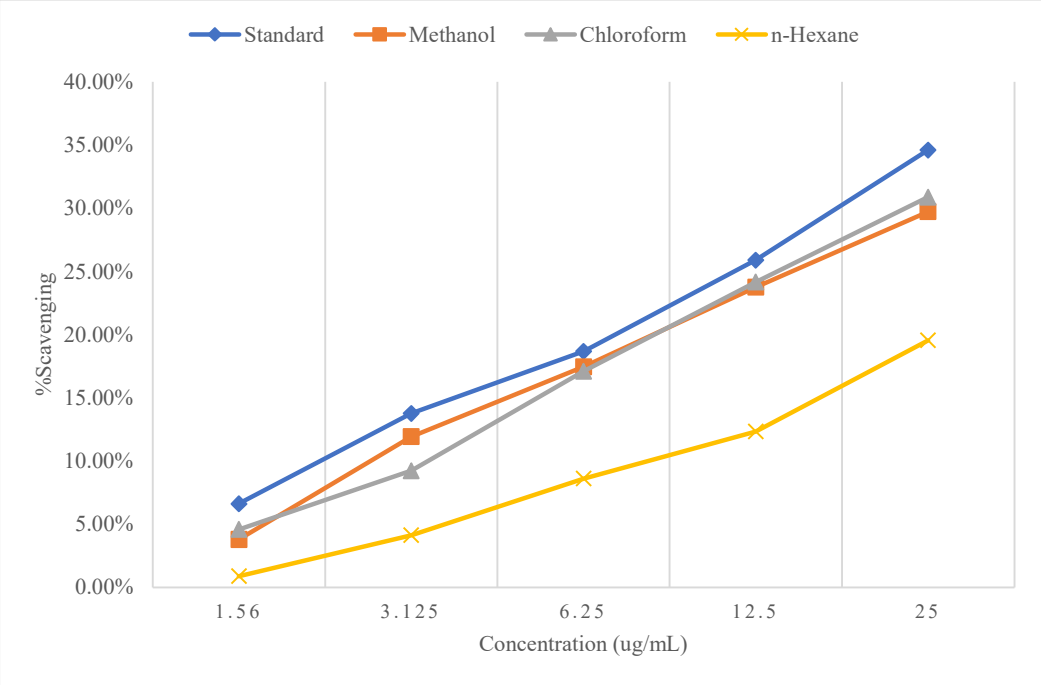

Figure 6: HRSA result comparison between standard, methanol, chloroform and n-Hexane extract

4.5 Total Antioxidant Capacity (TAC)

The total antioxidant capacity of the three *H. corymbosa* extracts was evaluated by the phosphomolybdenum method across five concentrations (6.25–100 µg/ml), with absorbance measured at 695 nm and ascorbic acid used as the positive control standard. The control (reagent blank) absorbance was 0.042. In this assay, higher absorbance directlyreflects greater electron-donating antioxidant capacity, as it corresponds to greater reduction of Mo(VI) to the green Mo(V)-phosphate complex.

4.5.1 Standard ascorbic acid result

Concentration(µg/ml)	Absorbance	Average
6.25	0.0863	0.084
	0.0781	
	0.0877	
12.5	0.1456	0.1446
	0.1394	
	0.1489	
25	0.2569	0.2627
	0.2536	
	0.2776	
50	0.3872	0.3778
	0.3753	
	0.371	
100	0.742	0.7291
	0.7128	
	0.7327	

Table 18: Ascorbic acid (Standard) results for TAC

4.5.2 Methanol extract result

Concentration(µg/ml)	Absorbance	Average
6.25	0.0675	0.0661
	0.066	
	0.065	
12.5	0.1482	0.1259
	0.1157	
	0.1138	
25	0.2145	0.2223
	0.2352	
	0.2173	
50	0.3158	0.3212
	0.3295	
	0.3183	
100	0.6124	0.6241
	0.6245	
	0.6356	

Table 19: Methanol extract test result for TAC

4.5.3 Chloroform extract result

Concentration(µg/ml)	Absorbance	Average
6.25	0.0682	0.0701
	0.0703	
	0.0719	
12.5	0.1498	0.1485
	0.1485	
	0.1474	
25	0.2583	0.2462
	0.2342	
	0.2462	
50	0.3717	0.3692
	0.3663	
	0.3698	
100	0.6524	0.6557
	0.6424	
	0.6723	

Table 20: Chloroform extract test result for TAC

4.5.4 n-Hexane extract result

Concentration(µg/ml)	Absorbance	Average
6.25	0.0237	0.0227
	0.0231	
	0.0213	
12.5	0.0738	0.0735
	0.0727	
	0.0742	
25	0.1635	0.1685
	0.1774	
	0.1648	
50	0.2345	0.229
	0.2183	
	0.2343	
100	0.4423	0.4459
	0.4639	
	0.4317	

Table 21: n-Hexane test result for TAC

4.5.5 Comparison

All three extracts demonstrated a clear and consistent concentration-dependent increase in absorbance at 695 nm, confirming dose-dependent electron-donating antioxidant capacity.[6] The chloroform extract produced the highest TAC values across all concentrations, reaching 0.6557 at 100 µg/ml. The crude methanol extract followed closely

with an absorbance of 0.6242 at the same concentration. The n-hexane extract recorded substantially lower values at all concentrations, reaching only 0.4459 at 100 µg/ml. All three extracts were weaker than the ascorbic acid standard (0.7291 at 100ug/ml).

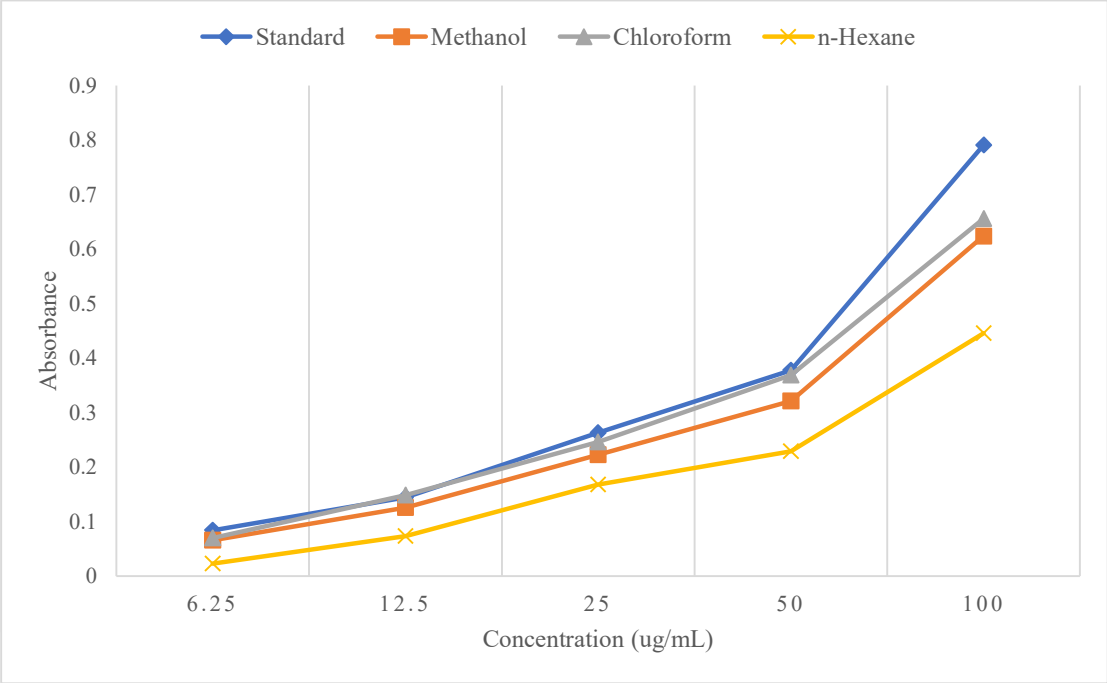

Figure 7: Comparison between test results of standard ascorbic acid, methanol, chloroform and n-hexane extract for TAC

4.6 Ferric reducing antioxidant power assay (FRAP)

The FRAP of the three extracts of *H.corymbosa* was assessed at five concentrations (6.25, 12.5, 25, 50, and 100 µg/ml), with ascorbic acid as the positive control standard. In this assay, a higher absorbance at 593 nm directly corresponds to greater reducing capacity, as it reflects a larger quantity of Fe(III) reduced to Fe(II)-TPTZ complex by the antioxidant components in the extract. Results are compared by absorbance across concentrations and expressed as a dose-dependent reducing activity profile.

4.6.1 Standard ascorbic acid results

Concentration(µg/ml)	Absorbance	Average
6.25	0.0843	0.0931
	0.0958	
	0.0993	
12.5	0.2802	0.2696
	0.2543	
	0.2743	
25	0.3597	0.3829
	0.3927	
	0.3965	
50	0.5121	0.524
	0.5362	
	0.5237	
100	0.6845	0.6509
	0.7133	
	0.5549	

Table 22: Ascorbic acid test result for FRAP

4.6.2: Methanol extract result

Concentration(µg/ml)	Absorbance	Average
6.25	0.0789	0.0614
	0.0781	
	0.0272	
12.5	0.1465	0.1605
	0.1958	
	0.1392	
25	0.2712	0.28
	0.3176	
	0.2513	
50	0.3081	0.2869
	0.3273	
	0.2253	
100	0.3869	0.371
	0.3505	
	0.3758	

Table 23: Methanol extract test result for FRAP

4.6.3: Chloroform extract result

Concentration(µg/ml)	Absorbance	Average
6.25	0.1032	0.1013
	0.0829	
	0.1178	
12.5	0.238	0.2384
	0.2203	
	0.257	
25	0.2816	0.3066
	0.3202	
	0.3182	
50	0.3995	0.3942
	0.3968	
	0.3865	
100	0.439	0.4449
	0.4312	
	0.4647	

Table 24: Chloroform extract test result for FRAP

4.6.4: n-Hexane extract result

Concentration(µg/ml)	Absorbance	Average
6.25	0.057	0.1003
	0.069	
	0.175	
12.5	0.1865	0.1218
	0.0906	
	0.0884	
25	0.1399	0.1506
	0.1708	
	0.1413	
50	0.1894	0.1878
	0.1732	
	0.2008	
100	0.2631	0.273
	0.2349	
	0.321	

Table 25: n-Hexane extract test result for FRAP

4.6.5: Comparison

All extracts demonstrated a concentration-dependent increase in absorbance at 593 nm, indicating progressive Fe(III) to Fe(II) reduction with increasing extract concentration. The ascorbic acid standard showed the highest reducing power at all tested concentrations, reaching an absorbance of 0.6509 at 100 µg/ml. Among the plant extracts, the chloroform fraction consistently produced the highest FRAP absorbance values across all concentrations, recording 0.4449 at 100 µg/ml. The methanol extract showed intermediate reducing capacity (0.371 at 100 µg/ml), while the n-hexane extract displayed the lowest FRAP values at all concentrations tested (0.2730 at 100 µg/ml).

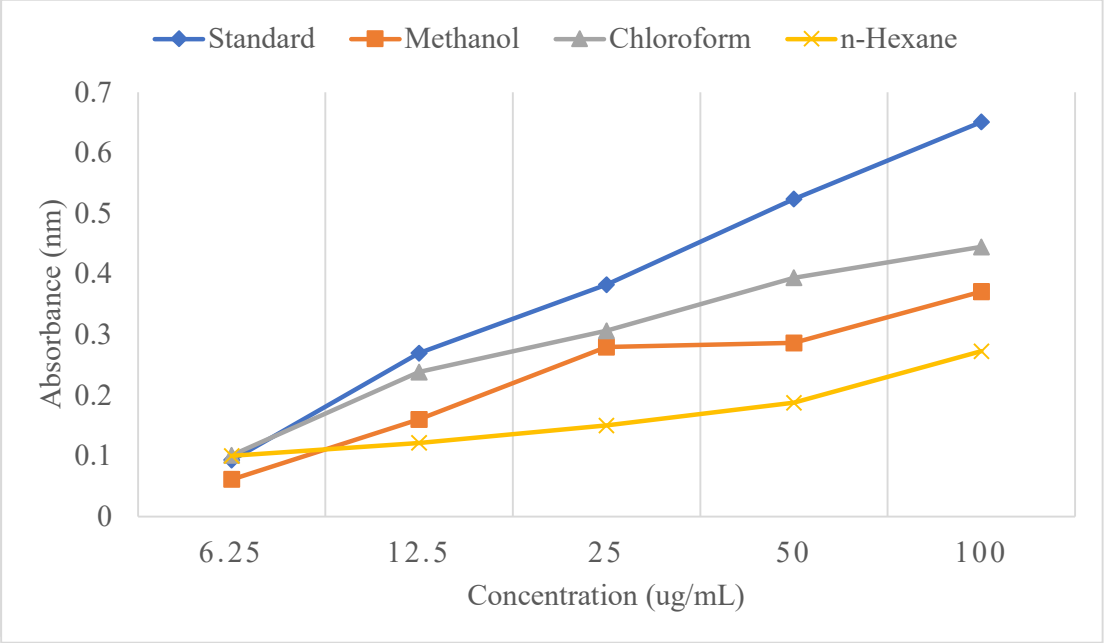

Figure 8: Comparison between test results of FRAP amongst ascorbic acid, methanol, chloroform, n-Hexane extract

4.7 Lipid Peroxidation Inhibition Test

The lipid peroxidation inhibition activity of the n-hexane, chloroform, and methanol extracts of *H.corymbosa* was evaluated using the thiobarbituric acid reactive substances (TBARS) method, with gallic acid as the positive reference standard. Percentage inhibition was determined at six concentrations (6.25, 12.5, 25, 50, 100, and 200 µg/ml) for all three extracts and the standard.

4.7.1 Standard gallic acid result

Concentration(µg/ml)	Absorbance	Average	%Scavenging
6.25	0.5918	0.5864	12.7%
	0.5762		
	0.5914		
12.5	0.497	0.484	27.9%
	0.4753		
	0.4797		
25	0.3383	0.3511	47.7%
	0.3538		
	0.3614		
50	0.3128	0.3241	51.7%
	0.3271		
	0.3324		
100	0.2564	0.2714	59.6%
	0.2944		
	0.2636		
200	0.2192	0.2106	68%
	0.2083		
	0.2045		

Table 26: Ascorbic acid test resulf for Lipid peroxidation Inhibition

4.7.2 Methanol extract test result

Concentration(µg/ml)	Absorbance	Average	%Scavenging
6.25	0.5941	0.5711	15%
	0.5171		
	0.6021		
12.5	0.5199	0.5056	24.7%
	0.5168		
	0.4802		
25	0.4787	0.4583	31.8%
	0.4334		
	0.4628		
50	0.4592	0.4487	33.2%
	0.4667		
	0.4202		
100	0.3541	0.3753	44.1%
	0.3957		
	0.3763		
200	0.2942	0.2719	59.5%
	0.2508		
	0.2709		

Table 27: Methanol extract test result for Lipid peroxidation inhibition

4.7.3 Chloroform extract test result

Concentration(µg/ml)	Absorbance	Average	%Scavenging
6.25	0.5876	0.5941	11.5%
	0.5935		
	0.6013		
12.5	0.5297	0.5102	24%
	0.4982		
	0.5028		
25	0.4182	0.436	35.1%
	0.4373		
	0.4526		
50	0.4272	0.4326	35.6%
	0.4215		
	0.4493		
100	0.3598	0.3828	43%
	0.3997		
	0.3891		
200	0.2673	0.2534	62.2%
	0.2472		
	0.2457		

Table 28: Chloroform extract test result for Lipid peroxidation inhibition

4.7.4 n-Hexane extract test result

Concentration(µg/ml)	Absorbance	Average	%Scavenging
6.25	0.6137	0.6053	9.9%
	0.5839		
	0.6185		
12.5	0.5982	0.5837	13.1%
	0.5592		
	0.5938		
25	0.5193	0.5006	25.5%
	0.4944		
	0.4882		
50	0.478	0.4669	30.5%
	0.4534		
	0.4695		
100	0.4567	0.4478	33.3%
	0.4545		
	0.4324		
200	0.3703	0.3837	42.9%
	0.4243		
	0.3567		

Table 29: n-Hexane extraction test result for Lipid peroxidation inhibition

4.7.5 Comparison

All three extract showed a concentration-dependent increase in lipid peroxidation inhibition across the tested range (6.25-200ug/ml). Gallic showed highest %I (68% at 200ug/ml). Amongst the sample extracts, chloroform extract ranks first (%I being 62.2%

at 200ug/ml), followed by methanol extract (59.5% at 200ug/ml) and n-Hexane showing the lowest inhibition rate (42.9% at 200ug/ml).

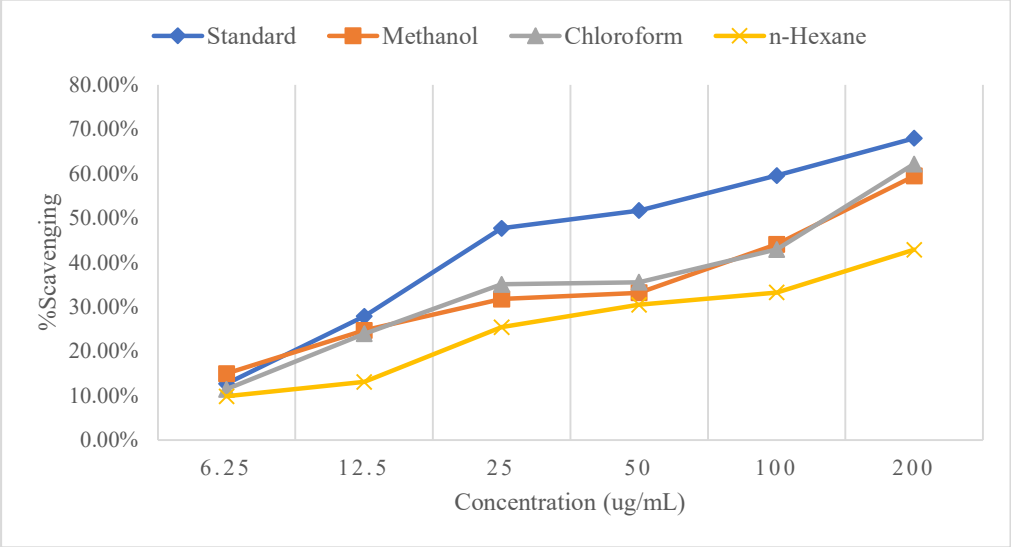

Figure 9: Comparison between standard gallic acid, methanol, chloroform, n-hexane extract for Lipid peroxidation inhibition

Chapter 5: Discussion

5.1 TPC result interpretation

Phenolic compounds are among the most widespread secondary metabolites in the plant kingdom, and their quantification serves as a foundational step in the phytochemical and pharmacological characterisation of any medicinal plant(Dai & Mumper, 2010). The Folin-Ciocalteu method,(Singleton & Rossi Jr, 1965), remains the universally accepted standard for total phenolic quantification in plant research. Its principle relies on the transfer of electrons from phenolic hydroxyl groups to the molybdotungstate reagent under alkaline conditions, producing a measurable blue colour at 760 nm.

The TPC values obtained in this study ranked as: chloroform (47.75 mg GAE/g) > methanol (42.01 mg GAE/g) > n-hexane (21.29 mg GAE/g). The finding that the chloroform fraction contains more total phenolics than the crude methanol extract, while initially counterintuitive, is directly explained by the sequential liquid-liquid fractionation procedure used in this study. Prior to TPC measurement, the crude methanol extract was partitioned first with n-hexane and then with chloroform. During the chloroform partitioning step, semi-polar phenolic compounds, particularly aglycone flavonoids and low-polarity phenolic acids, that were originally dissolved in the methanol extract preferentially migrated into the chloroform phase, thereby concentrating them there(Sultana et al., 2009). The chloroform fraction is therefore a phenolic-enriched sub-fraction of the original methanol extract, and its higher TPC relative to the remaining methanol fraction is a predictable outcome of this partitioning chemistry(Stalikas, 2007).

This fractionation-enrichment phenomenon has been documented in numerous studies on Rubiaceae and other plant families. For example, Stalikas (2007) demonstrated that chloroform partitioning consistently concentrates aglycone phenolics relative to the parent polar extract, precisely because the reduced polarity of aglycones relative to their glycosylated counterparts drives preferential partitioning into the organic phase(Stalikas, 2007).

Contextualising the TPC data against the published literature reveals that *H. corymbosa* is a moderately to well-phenolic medicinal plant. Comparative values from other Rubiaceae species include methanol extract TPC values of approximately 25–45 mg GAE/g for *Morinda citrifolia* fruit(Zin et al., 2002). The values reported here for all three fractions fall within or above this reference range, supporting the pharmacological relevance of *H. corymbosa* as a source of bioactive phenolic compounds.

The n-hexane fraction's TPC (21.29 mg GAE/g) is low but non-negligible. Since aromatic hydroxyl-bearing phenolic structures are poorly soluble in non-polar aliphatic solvents(Sultana et al., 2009), the measurable TPC in the n-hexane fraction likely reflects a small quantity of very low-polarity phenolic aglycones co-extracted alongside waxes and terpenoids, and possibly some contribution from tocopherols which also reduce the FC reagent to a modest degree(Decker, 1998).The gallic acid calibration curve performed well ($R^2 = 0.9827$), meeting the standard acceptance criterion of $R^2 \geq 0.98$ that is widely applied in quantitative phytochemical studies(Huang et al., 2005).

5.2 TFC result interpretation

Flavonoids constitute the most pharmacologically investigated subclass of plant phenolics, with over 9,000 distinct structures identified across the plant kingdom(Middleton Jr et al., 2000). Their characteristic C6-C3-C6 diphenylpropane skeleton comprising two benzene rings linked via an oxygenated three-carbon bridge confers both structural diversity and antioxidant potency. Antioxidant mechanisms attributed to flavonoids include: direct radical scavenging through HAT and SET; chelation of redox-active transition metals (Fe^{2+}, Cu^{+}) that catalyse free radical generation via Fenton-type reactions; inhibition of pro-oxidant enzymes such as xanthine oxidase; and activation of endogenous antioxidant pathways through Nrf2-mediated gene regulation(Williams et al., 2004). Measuring TFC therefore provides mechanistically specific context for the antioxidant activity observed across the assay panel.

The aluminium chloride method was selected for TFC determination because $AlCl_3$ forms stable, intensely yellow chelate complexes exclusively with flavonoids(Chang et al., 2002). This structural selectivity distinguishes the $AlCl_3$ method from the FC method: while TPC responds to all phenolics and many other reductants, TFC specifically captures the aluminium-chelatable flavonoid subclass(Zhishen et al., 1999). Quercetin was used as the standard because it is one of the most widely distributed flavonols in nature, possesses all

the chelation-relevant structural features and forms a well-characterised and reproducible complex with $AlCl_3$(Kumar & Pandey, 2013).

The TFC ranking, chloroform (32.86 QE/g) > methanol (28.83 mg QE/g) > n-hexane (13.93 mg QE/g) is identical to the TPC ranking and reflects the same underlying fractionation chemistry. The chloroform fraction's dominance in TFC results from the preferential partitioning of aglycone flavonoids into the chloroform phase during liquid-liquid fractionation(Stalikas, 2007; Sultana et al., 2009). Aglycone flavonoids lack sugar substituents, which significantly increases their lipophilicity relative to glycosylated forms and drives their migration from the aqueous methanol phase into the organic chloroform phase(Manach et al., 2004). The chloroform fraction's superior TFC therefore reflects both genuine flavonoid enrichment and an assay-response advantage attributable to the aglycone structural form.

The crude methanol extract's TFC (28.83 mg QE/g) is lower than the chloroform fraction despite it's probable that the methanol fraction contained a greater total quantity of flavonoids before fractionation. This can be understood by knowing what happens during fractionation: after liquid-liquid partitioning, the lipophilic flavonoids migrate into the chloroform layer, leaving the residual methanol fraction enriched in the more polar flavonoids(Stalikas, 2007).The n-hexane fraction's TFC (13.9 mg QE/g), while the lowest of the three, is not negligible and warrants mechanistic discussion. Non-polar solvents are not generally expected to extract flavonoids, as these compounds require at least two to three free hydroxyl groups for their characteristic antioxidant reactivity(Sultana et al., 2009). However, a specific subclass of flavonoids,

the polymethoxylated flavonoids (PMFs), have substantially reduced polarity and can be extracted by semi-polar or even weakly non-polar solvents(Harborne, 1998). PMFs have been identified as characteristic constituents of several Rubiaceae genera(Lin et al., 1995), and their possible presence in *H. corymbosa* provides a plausible explanation for the measurable flavonoid content detected in the n-hexane fraction. Definitive confirmation would require chromatographic isolation (e.g., HPLC-DAD or GC-MS) and spectroscopic identification, which represent important directions for follow-up investigation.

5.3 DPPH result interpretation

The DPPH radical scavenging assay is among the most widely employed and best-validated methods for the preliminary screening of antioxidant activity in plant extracts(Blois, 1958). In this assay, antioxidants reduce the stable, violet-coloured DPPH radical to the pale yellow DPPH-H molecule by donating either a hydrogen atom (HAT mechanism) or a single electron (SET mechanism), with the resultant decrease in absorbance at 517 nm being proportional to antioxidant potency(Brand-Williams et al., 1995).

The methanol extract data is the compressed, showing shallow dose-response curve: inhibition was already at 39.0 at the lowest tested concentration (3.125 µg/ml) and rose to only 57.2% at 100 µg/ml. This plateau-like response may reflect the presence of multiple antioxidant compounds acting through different mechanisms, including radical scavenging, metal chelation, and reducing power, rather than a single dominant scavenging mechanism(Bondet et al., 1997).

The chloroform extract showed a more gradual and linear dose-response (30.2% at 3.125 µg/ml rising to 62.4% at 100 µg/ml), which is mechanistically consistent with a fraction enriched in aglycone flavonoids and semi-polar terpenoids that have more homogeneous and moderate reaction kinetics with DPPH(Rice-Evans et al., 1996).

The n-hexane extract showed the weakest DPPH activity, with a notable plateau in the dose-response curve above 25 µg/ml (31.7% at 25 µg/ml vs. 36.16% at 100 µg/ml, only 4.46 percentage points gained across a four-fold concentration increase). This plateau pattern strongly suggests that the antioxidant compounds in the n-hexane fraction do not primarily operate through the hydrogen atom donation or electron transfer mechanisms captured by DPPH(Prior et al., 2005).

5.4 HRSA result interpretation

The hydroxyl radical (•OH) is the most damaging of the biologically relevant reactive oxygen species, reacting at near-diffusion-controlled rates with virtually all organic molecules like proteins, lipids, sugars, and DNA without selectivity as previously discoused.

None of the three *H. corymbosa* extracts nor the BHT standard reached 50% hydroxyl radical inhibition within the tested concentration range (1.56–25 µg/ml). This result must be interpreted in its experimental context: the concentration range selected for this assay was considerably lower than those used for the DPPH and TAC assays. This uniformly modest inhibition across all samples, including the standard, indicates that

the concentration range was conservative for this particular assay system and should be extended in future work to at least 100–200 µg/ml to enable more than 50% inhibition.

Despite not reavhing 50% inhibition the data has significance. All three extracts displayed consistent, linear concentration-dependent increases in % inhibition. At the highest tested concentration of 25 µg/ml, the rank order was: chloroform (30.9%) > methanol (29.74%) > n-hexane (19.56%). The near-identical performance of the chloroform and methanol extracts in this assay is noteworthy and differs from their divergence in the DPPH assay. This convergence likely reflects the fact that the HRSA assay operates in a fully aqueous phosphate buffer at physiological pH (7.4), a condition under which both polar flavonoid glycosides (dominant in the methanol fraction) and aglycone flavonoids (dominant in the chloroform fraction) can contribute through metal chelation, a mechanism that is independent of solvent-phase hydrogen atom donation(Williams et al., 2004). Metal chelation is especially important in the HRSA test because the chemical reaction that produces harmful hydroxyl radicals needs free iron (Fe^{2+}) to work. Certain flavonoids can bind to and trap this iron, effectively shutting down the reaction before any harmful radicals are even formed. This iron-trapping ability is found in flavonoids that have specific structural features, such as quercetin, luteolin, and kaempferol(Williams et al., 2004). Both types of these flavonoids have been found in plant species closely related to H. corymbosa(Lin et al., 1995), and their presence in both the chloroform and methanol fractions offers a likely explanation for why both fractions showed similar levels of hydroxyl radical-fighting activity in the test.

The n-hexane extract showed substantially weaker HRSA at all concentrations. This is expected because the HRSA assay takes place in an aqueous medium, and the predominantly lipophilic compounds of the n-hexane fraction have limited aqueous solubility and therefore reduced access to the metal ions and radical species in the reaction solution(Spigno et al., 2007).

5.5 TAC result interpretation

The phosphomolybdenum Total Antioxidant Capacity (TAC) assay measures the cumulative electron-donating capacity of all antioxidant species present in an extract through the single electron transfer (SET) mechanism, under strongly acidic conditions at elevated temperature(Prieto et al., 1999). Unlike the DPPH assay, which operates in a methanolic medium favouring polar compound solubility, the TAC assay is aqueous and captures contributions from a broad range of electron-donating compounds, including ascorbic acid, phenolics, α-tocopherol, and carotenoids(Umamaheswari & Chatterjee, 2008). This broad detection scope makes the TAC assay particularly valuable as a corroborating measure of overall antioxidant capacity when used alongside more mechanistically specific assays such as DPPH and HRSA.

The TAC ranking at 100 µg/ml: chloroform (0.6557) > methanol (0.6241) > n-hexane (0.4459) mirrors the pattern observed TPC and TFC in this study, where chloroform also ranked first. This convergent pattern across TPC, TFC, and TAC provides strong evidence that the semi-polar phenolic and flavonoid compounds enriched in the chloroform fraction are the primary drivers of electron-donating antioxidant capacity in this plant(Crozier et al., 2009).

The n-hexane extract, while substantially lower in TAC than the other two fractions, nevertheless demonstrated a consistent and reproducible dose-response increase from 0.0227 at 6.25 µg/ml to 0.4459 at 100 µg/ml. This confirms the presence of modest electron-donating capacity in the lipophilic fraction, most likely attributable to carotenoids and/or tocopherols(Decker, 1998).

Taken together, the TAC results for *H. corymbosa* lead to the following conclusions: both the chloroform and methanol fractions have strong antioxidant capacity, coming close to matching the performance of the ascorbic acid standard at 100 µg/ml; the chloroform fraction performs slightly better, which aligns with its higher levels of phenols and flavonoids; and all three extracts show real, dose-dependent antioxidant activity through the electron-donating mechanism. These findings support and strengthen the results from the DPPH and HRSA tests, confirming that H. corymbosa is a credible natural source of antioxidants that works through multiple different pathways.

5.6 FRAP assay result interpretation

The assay operates primarily through the single electron transfer (SET) mechanism, and the FRAP value therefore reflects the reducing power of all electron-donating species present in the extract(Apak et al., 2007). This makes FRAP a useful complement to the DPPH and hydroxyl radical scavenging assays provides a more complete picture of the total antioxidant capacity when used alongside these assays(Huang et al., 2005).

All three extracts of *H. corymbosa* exhibited concentration-dependent increases in Fe(III) reducing capacity, confirming the presence of electron-donating antioxidant compounds in each fraction. The ascorbic acid standard showed the strongest reducing power at all concentrations, which is expected given ascorbic acid's well-characterised two-electron donating mechanism and high molar reducing capacity(Buettner, 1993).Among the plant extracts, the chloroform fraction consistently produced the highest FRAP absorbance values at all concentrations tested, reaching 0.4449 at 100 µg/ml, compared to 0.371 for the methanol extract and 0.273 for the n-hexane extract at the same concentration.

The superior FRAP activity of the chloroform fraction can be attributed to its higher total phenolic content (TPC) and total flavonoid content (TFC) relative to the methanol extract. Phenolic compounds are well-established electron donors, and their capacity to reduce Fe^{3+} to Fe^{2+} in the FRAP assay is directly proportional to the number of hydroxyl groups available on their aromatic rings(Benzie & Strain, 1996). Flavonoids, as a major subclass of phenolics, contribute significantly to this reducing power, particularly aglycone forms, which are more readily found in non-polar fractions such as chloroform as previously discussed. Since aglycones lack the sugar attachments present in glycosides, they possess greater structural accessibility for electron donation, making them more efficient reducing agents in the FRAP system(Hollman et al., 1999).

The n-hexane extract demonstrated the lowest FRAP values across all concentrations, consistent with the expected paucity of polar electron-donating phenolics in this lipophilic fraction(Dai & Mumper, 2010). Whilst carotenoids and tocopherols can

theoretically contribute to ferric reduction, their concentrations in the n-hexane fraction of a small herbaceous plant such as *H. corymbosa* appear to be insufficient to generate absorbance values approaching those of the other two fractions. The ranking of FRAP activity (chloroform > methanol > n-hexane) is broadly consistent with the pattern observed in DPPH, HRSA assay and reinforces the conclusion that the semi-polar fraction of *H. corymbosa* is particularly rich in electron-donating antioxidant compounds.

5.7 Lipid peroxidation inhibition assay result interpretation

Lipid peroxidation is a well-recognised process of oxidative damage in which free radicals attack the fatty acid chains of cell membranes, triggering a self-propagating chain reaction that ultimately leads to cell membrane destruction and tissue injury(Niki, 2009). The ability of a plant extract to inhibit this process is therefore considered a highly meaningful indicator of its potential to protect biological systems from oxidative stress-related damage(Duh et al., 1999). In the present study, the lipid peroxidation inhibition activity of the three fractions of H. corymbosa was evaluated using the thiobarbituric acid reactive substances (TBARS) method, with gallic acid serving as the reference standard. The results are expressed as percentage inhibition (%I) across a concentration range of 6.25 to 200 µg/ml.

The standard gallic acid demonstrated a steady and progressive increase in inhibition throughout the tested concentration range, rising from 12.7% at 6.25 µg/ml to 68.0% at 200 µg/ml. This consistent, concentration-dependent pattern is characteristic of a potent

antioxidant compound and served as a reliable benchmark for evaluating the performance of the plant extracts(Benzie & Strain, 1996).

Among the three plant extracts, the methanol and chloroform fractions exhibited broadly comparable and notably stronger lipid peroxidation inhibition activity compared to the n-hexane fraction across most concentrations tested. The methanol extract showed the highest inhibition at the lower concentration range, recording 15.0% at 6.25 µg/ml and 24.7% at 12.5 µg/ml, slightly outperforming the chloroform fraction, which recorded 11.5% and 24.0% at the same concentrations respectively. However, as the concentration increased, the chloroform fraction gradually closed this gap, recording 35.1% at 25 µg/ml compared to 31.8% for the methanol extract at the same concentration. At the highest tested concentration of 200 µg/ml, the chloroform fraction ultimately surpassed the methanol extract, achieving 62.2% inhibition compared to 59.5%, suggesting that its antioxidant compounds may exert greater overall potency at higher doses.

An interesting pattern was observed when comparing the methanol and chloroform fractions: at lower concentrations, the methanol extract performed slightly better, but at higher concentrations, the chloroform fraction took the lead. This shift can be explained by the different types of compounds present in each fraction. At lower concentrations, the compounds in the methanol extract, which are more water-friendly, dissolve and interact more easily in the water-based test environment, allowing them to act quickly and

effectively(Heim et al., 2002). However, at higher concentrations, the compounds in the chloroform fraction, which are simpler in structure because they lack the sugar attachments found in the methanol extract's compounds, appear to be more efficient at stopping lipid oxidation(Hollman et al., 1999). In simple terms, the absence of these sugar attachments allows the chloroform compounds to donate electrons more freely and effectively. This explanation is further backed by the fact that the chloroform fraction contains higher levels of total phenols and flavonoids compared to the methanol extract, and phenolic compounds are well known for their ability to interrupt and stop the chain reaction of lipid oxidation before it can spread further(Rice-Evans et al., 1996).

In contrast, the n-hexane fraction consistently demonstrated the weakest lipid peroxidation inhibition across all concentrations, with %I values of 9.9% at 6.25 µg/ml increasing only to 42.9% at 200 µg/ml. Even at the highest concentration tested, the n-hexane fraction failed to reach the inhibition levels achieved by the methanol and chloroform fractions at much lower doses. This poor performance is consistent with the expected low phenolic and flavonoid content of a non-polar hexane fraction, which predominantly extracts non-polar compounds such as waxes, sterols, and terpenoids that generally possess limited capacity for lipid radical chain-breaking activity(Azwanida, 2015).

Overall, the lipid peroxidation inhibition results confirm that H. corymbosa possesses meaningful protective activity against membrane lipid oxidation, with the methanol and

chloroform fractions being the primary contributors to this activity. Although neither fraction reached the inhibition levels of gallic acid at equivalent concentrations, their performance across the full concentration range, particularly the chloroform fraction at higher doses, demonstrates that H. corymbosa contains bioactive compounds capable of meaningfully suppressing lipid peroxidation. These findings complement and reinforce the results obtained from the DPPH, HRSA, and FRAP assays, collectively building a strong case for the antioxidant potential of this plant across multiple mechanistic pathways.

Chapter 6: Conclusion

The present study provides a systematic and comprehensive evaluation of the antioxidant potential of *H.corymbosa* (L.) Lam. sourced from Bangladesh, assessed across three solvent fractions using a broad panel of complementary in vitro assays. The findings collectively confirm that this plant possesses meaningful and genuine antioxidant activity, lending scientific credibility to its long-standing use in traditional medicine systems across South and Southeast Asia for conditions closely associated with oxidative stress, including fever, liver disorders, and inflammatory conditions.

Among the three fractions tested, the chloroform fraction consistently emerged as the most potent, demonstrating the highest total phenolic content (TPC: 47.75 mg GAE/g) and total flavonoid content (TFC: 32.85 mg QE/g), and producing the strongest or near-strongest results across the DPPH radical scavenging, hydroxyl radical scavenging, total antioxidant capacity, ferric reducing antioxidant power, and lipid peroxidation inhibition assays. This superior performance is attributed to the enrichment of lipophilic aglycone flavonoids in the chloroform fraction following liquid-liquid partitioning, which, due to the absence of sugar attachments, possess greater structural efficiency as electron donors and free radical scavengers. The methanol extract also demonstrated substantial antioxidant activity across all assays, performing closely behind the chloroform fraction in most tests and occasionally surpassing it at lower concentrations, particularly in the lipid peroxidation inhibition assay. This behaviour is explained by

the presence of polar flavonoid glycosides and phenolic acids in the methanol fraction, which are more readily soluble and accessible in water-based assay environments. The n-hexane fraction consistently showed the weakest activity across all assays, which is consistent with the low phenolic and flavonoid content expected from a highly non-polar extraction solvent that predominantly captures waxes, oils, and terpenoids rather than phenolic antioxidants.

The convergence of results across mechanistically distinct assays, covering hydrogen atom transfer, electron donation, metal chelation, and lipid radical chain-breaking mechanisms, further strengthens the conclusion that *H. corymbosa* exerts its antioxidant effects through multiple complementary pathways rather than a single mechanism. This multi-pathway activity is particularly significant from a pharmacological standpoint, as antioxidants that work through several mechanisms simultaneously are generally considered more effective in protecting biological systems from oxidative damage than those acting through a single pathway alone.

While the results of this study are promising, it is important to acknowledge that in vitro antioxidant assays, though valuable as initial screening tools, do not directly predict biological activity in living systems. The bioavailability, metabolism, and in vivo efficacy of the bioactive compounds identified in *H. corymbosa* remain to be established through further investigation. Future studies should therefore focus on isolating and identifying the specific phenolic and flavonoid compounds responsible for the observed activity,

evaluating their bioavailability and stability under physiological conditions, and conducting in vivo and cell-based studies to determine their true therapeutic potential.

In summary, this study successfully demonstrates that *H.corymbosa* is a pharmacologically credible source of natural antioxidants, with the chloroform fraction identified as the most active fraction and phenolic compounds confirmed as the key contributors to its antioxidant capacity. These findings provide a solid scientific foundation for future pharmacological research into this underexplored medicinal plant and support its continued investigation as a potential source of natural therapeutic agents.

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