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“ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC CONTENT AND TOTAL FLAVONOID CONTENT OF ETHANOLIC LEAF EXTRACT OF *Psidium guajava L.*”

A dissertation submitted to the Department of Pharmacy, Independent University, Bangladesh in partial fulfillment of the degree of Bachelor of Pharmacy

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DECLARATION

I hereby declare that the project work entitled “Antioxidant Activity, Total Phenolic Content and Total Flavonoid Content of Ethanolic Leaf Extract of *Psidium guajava* L.” is an original work carried out by me in the Pharmaceutical Sciences Laboratory, Department of Pharmacy, Independent University, Bangladesh (IUB), under the supervision of Dr. Nasrin Akhter, Associate Professor & Head, Department of Pharmacy, Independent University, Bangladesh.

I further declare that this work has not been submitted, in whole or in part, for the award of any other degree or diploma at this or any other institution. All sources of information and literature used in this report have been duly acknowledged and referenced.

Submitted by

.....

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ABSTRACT

Guava (*Psidium guajava* L., family Myrtaceae) leaves are widely used in traditional medicine for the management of diarrhoea, diabetes, inflammation and infections, and they are known to be rich in polyphenols. The present study aimed to evaluate the *in vitro* antioxidant activity and to determine the total phenolic and total flavonoid contents of the ethanolic leaf extract of *P. guajava*.

Guava leaves collected from Birulia, Savar, Dhaka, were shade-dried and powdered, and 400 g of the powder was extracted with 800 mL of 98% ethanol by cold maceration for 3 days. The filtrate was concentrated under reduced pressure using a rotary evaporator to obtain 20.22 g of crude extract (yield 5.06% w/w). The antioxidant activity of the extract was evaluated by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay using ascorbic acid as the standard. The total phenolic content (TPC) was determined by the Folin–Ciocalteu method using gallic acid as the standard, and the total flavonoid content (TFC) was determined by the aluminium chloride colorimetric method using quercetin as the standard.

The extract showed concentration-dependent DPPH radical scavenging activity, with inhibition increasing from 13.51% at 12.5 µg/mL to 56.32% at 200 µg/mL, and an IC_{50} value of 169.04 µg/mL compared with 13.06 µg/mL for ascorbic acid. The extract contained a high total phenolic content of 471.17 ± 50.40 mg gallic acid equivalents (GAE)/g and a total flavonoid content of 132.01 ± 11.17 mg quercetin equivalents (QE)/g of dry extract. The calibration curves of gallic acid (R^2 = 0.9943) and quercetin (R^2 = 0.9944) showed good linearity. At matched concentrations, DPPH inhibition increased in parallel with the phenolic (r = 0.971) and flavonoid (r = 0.997) contents, suggesting that these constituents contribute to the antioxidant activity of the extract.

The findings indicate that guava leaves, an abundant and inexpensive agricultural by-product in Bangladesh, are a rich source of natural phenolic antioxidants, and they provide scientific support for the traditional medicinal use of the plant. Further studies involving other antioxidant assays, isolation and identification of the active compounds, and cell-based and *in vivo* evaluation are recommended to establish its therapeutic potential.

Keywords: *Psidium guajava*; guava leaf; antioxidant activity; DPPH radical scavenging; IC_{50}; total phenolic content; total flavonoid content; Folin–Ciocalteu; aluminium chloride; ethanolic extract.

CHAPTER 1
INTRODUCTION

1.1 Background of the Study

Medicinal plants have been used to prevent and treat human diseases since ancient times, and they continue to be an essential part of health care in many parts of the world. According to the World Health Organization, traditional and complementary medicine, much of which is based on plant materials, remains widely used in the majority of its member states *(World Health Organization, 2019)*. The growing use of herbal products has, however, also increased the need for scientific evidence regarding their efficacy, quality and safety *(Ekor, 2014)*. Natural products have historically been one of the richest sources of new drug leads, and a substantial proportion of approved small-molecule drugs are natural products, their derivatives or compounds inspired by them *(Newman & Cragg, 2020; Atanasov et al., 2021)*.

One of the most important biological properties of plant-derived compounds is their antioxidant activity. Reactive oxygen species (ROS) and other free radicals are continuously produced in the human body during normal metabolism. When their production exceeds the capacity of the body's antioxidant defence system, a condition known as oxidative stress develops, which damages lipids, proteins and DNA and has been linked to cancer, diabetes mellitus, cardiovascular disease, neurodegenerative disorders, chronic inflammation and ageing *(Valko et al., 2007; Pham-Huy et al., 2008; Reuter et al., 2010)*. Dietary and medicinal plants are rich sources of natural antioxidants, particularly phenolic compounds and flavonoids, which can scavenge free radicals, chelate pro-oxidant metal ions and support endogenous antioxidant defences *(Pandey & Rizvi, 2009; Dai & Mumper, 2010)*.

Psidium guajava L. (guava), a member of the family Myrtaceae, is an important fruit tree cultivated throughout tropical and subtropical regions, including India, Pakistan, Indonesia, Bangladesh and South America *(Kumar et al., 2021)*. Although the plant is best known for its nutritious fruit, its leaves have a long history of use in folk medicine for the treatment of diarrhoea, dysentery, gastrointestinal disorders, diabetes, cough, oral and dental problems, wounds and skin infections *(Gutiérrez et al., 2008; Díaz-de-Cerio et al., 2017)*. Guava leaves are rich in polyphenols, including flavonoids (such as quercetin and its glycosides), phenolic acids and tannins, as well as triterpenoids and essential oils, which are believed to be responsible for many of their pharmacological properties *(Gutiérrez et al., 2008; Kumar et al., 2021)*.

In Bangladesh, guava (locally known as *peyara*) is one of the most widely grown and popular fruits, and its leaves are abundantly available, inexpensive and largely discarded as agricultural by-products. Scientific evaluation of the antioxidant potential and phenolic composition of locally obtained guava leaves could therefore help to add value to this underutilised resource and provide a scientific basis for its traditional uses. In the present study, the ethanolic extract of *P. guajava* leaves was evaluated for its free radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and its total phenolic content (TPC) and total flavonoid content (TFC) were determined using the Folin–Ciocalteu and aluminium chloride colorimetric methods, respectively *(Singleton & Rossi, 1965; Brand-Williams et al., 1995; Chang et al., 2002)*.

1.2 Free Radicals, Reactive Oxygen Species and Oxidative Stress

1.2.1 Free Radicals and Reactive Oxygen Species (ROS)

A free radical is any atom or molecule that contains one or more unpaired electrons in its outer orbital and is capable of independent existence. Because of the unpaired electron, free radicals are highly unstable and reactive, and they tend to attack nearby molecules to achieve stability, thereby initiating chain reactions *(Halliwell, 2006; Lobo et al., 2010)*. Reactive oxygen species (ROS) include both free radicals, such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$) and peroxyl radical ($ROO^{\bullet}$), and non-radical oxidants such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Reactive nitrogen species (RNS), such as nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$), are closely related oxidants *(Valko et al., 2007; Birben et al., 2012)*.

Free radicals are generated from both endogenous and exogenous sources. Endogenous sources include the mitochondrial electron transport chain, activated phagocytes (through NADPH oxidase), xanthine oxidase, cytochrome P450 enzymes and peroxisomes, whereas exogenous sources include ultraviolet and ionising radiation, air pollutants, cigarette smoke, alcohol, heavy metals, certain drugs and pesticides *(Pham-Huy et al., 2008; Birben et al., 2012)*. At low or moderate concentrations, ROS play useful physiological roles in cell signalling, immune defence against pathogens and the regulation of gene expression *(Valko et al., 2007; Sies, 2015)*.

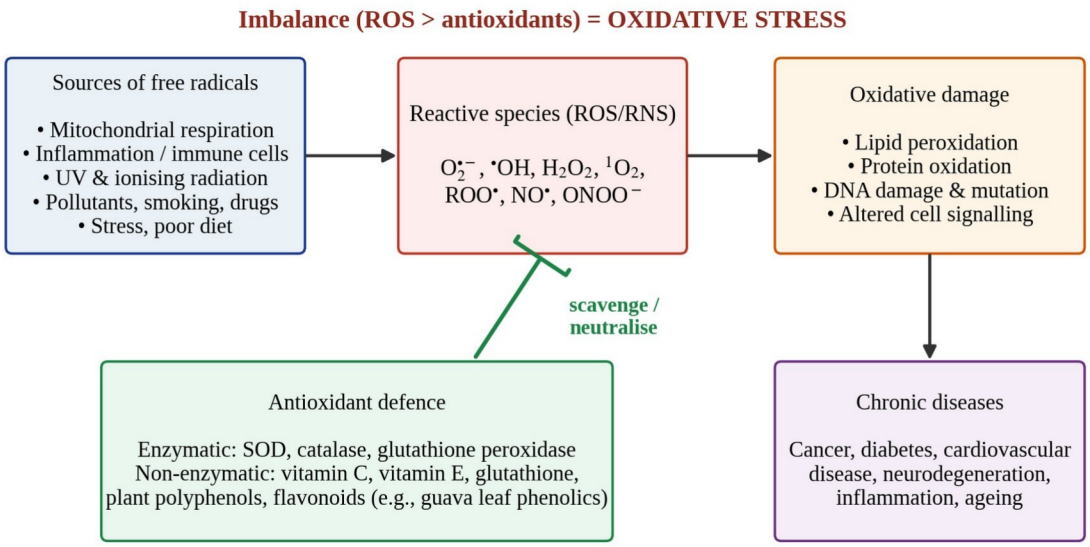


Figure 1.1: Sources of free radicals, oxidative damage and the protective role of antioxidants.

1.2.2 Oxidative Stress and Mechanism of Oxidative Damage

Oxidative stress is defined as an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage *(Sies, 2015)*. Under such conditions, excess ROS attack all major classes of biomolecules *(Valko et al., 2007; Birben et al., 2012)*:

- **Lipid peroxidation:** ROS abstract hydrogen atoms from polyunsaturated fatty acids of cell membranes, initiating a self-propagating chain reaction that produces lipid peroxyl radicals and reactive aldehydes such as malondialdehyde and 4-hydroxynonenal, leading to loss of membrane integrity.
- **Protein oxidation:** oxidation of amino acid side chains causes protein carbonylation, cross-linking and fragmentation, resulting in loss of enzyme and receptor function.
- **DNA damage:** the hydroxyl radical reacts with DNA bases and the sugar–phosphate backbone, producing oxidised bases (e.g., 8-hydroxy-2′-deoxyguanosine), strand breaks and mutations that may contribute to carcinogenesis.

1.2.3 Oxidative Stress and Human Diseases

Sustained oxidative stress has been implicated in the initiation and progression of numerous chronic diseases, including cancer, atherosclerosis and other cardiovascular diseases, diabetes mellitus and its complications, neurodegenerative disorders such as

Alzheimer's and Parkinson's diseases, rheumatoid arthritis, cataract and the ageing process *(Rahman, 2007; Pham-Huy et al., 2008)*. Oxidative stress and chronic inflammation are closely interlinked: activated inflammatory cells release large amounts of ROS, while ROS activate transcription factors such as nuclear factor-κB that amplify inflammatory signalling, creating a vicious cycle that favours tumour development *(Reuter et al., 2010)*. Consequently, antioxidants that can limit oxidative damage are of considerable interest for the prevention and management of these conditions.

1.3 Antioxidants

An antioxidant may be defined as any substance that, when present at a low concentration compared with that of an oxidisable substrate, significantly delays or prevents the oxidation of that substrate *(Halliwell, 2006)*. Antioxidants protect cells by scavenging free radicals, breaking radical chain reactions, chelating transition metal ions that catalyse radical formation, quenching singlet oxygen and repairing or removing damaged molecules *(Gulcin, 2020)*.

1.3.1 Classification of Antioxidants

Antioxidants are commonly classified according to their nature and mode of action *(Birben et al., 2012; Gulcin, 2020)*:

- **Enzymatic antioxidants:** superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase, which form the primary endogenous defence.
- **Non-enzymatic antioxidants:** glutathione, vitamin C (ascorbic acid), vitamin E (tocopherols), carotenoids, uric acid and plant polyphenols, including flavonoids.
- **Primary (chain-breaking) antioxidants:** compounds that donate a hydrogen atom or an electron to free radicals and convert them into more stable products.
- **Secondary (preventive) antioxidants:** compounds that act indirectly, for example by chelating pro-oxidant metal ions, scavenging oxygen or decomposing peroxides.
- **Natural and synthetic antioxidants:** natural antioxidants are obtained from plants and other biological sources, whereas synthetic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate.

1.3.2 Importance of Natural Antioxidants

Synthetic antioxidants such as BHA and BHT are effective and inexpensive and have been widely used in the food and pharmaceutical industries. However, concerns about their possible toxicity and carcinogenicity at high doses, together with consumer preference for natural ingredients, have stimulated the search for safe and effective antioxidants of natural origin *(Lourenço et al., 2019)*. Plant-derived antioxidants are often consumed as part of the normal diet, and many of them possess additional beneficial activities, such as anti-inflammatory, antimicrobial and anticancer effects *(Pandey & Rizvi, 2009; Shahidi & Ambigaipalan, 2015)*.

1.3.3 Increasing Interest in Plant-Based Bioactive Compounds

Plants synthesise a remarkable diversity of secondary metabolites, including phenolic compounds, flavonoids, terpenoids and alkaloids, which help them to cope with environmental stresses and defend against pathogens and herbivores. Many of these metabolites have been shown to exert antioxidant and other health-promoting effects in humans *(Dai & Mumper, 2010; Tungmunnithum et al., 2018)*. Advances in analytical techniques and growing interest in functional foods and nutraceuticals have renewed attention on plant materials, especially inexpensive and abundant ones such as leaves and agricultural by-products, as sources of natural antioxidants *(Atanasov et al., 2021; Kumar et al., 2021)*.

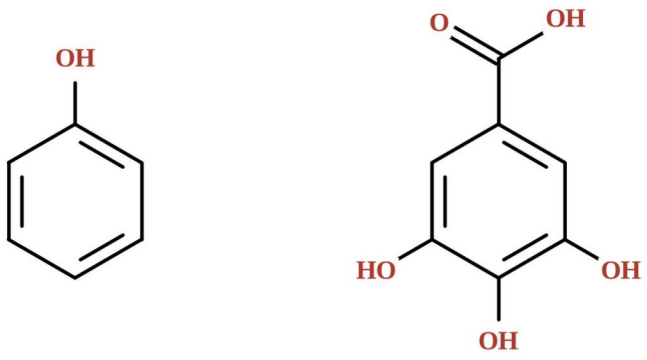
1.4 Phenolic Compounds

1.4.1 Definition and Classification

Phenolic compounds are secondary metabolites that possess an aromatic ring bearing one or more hydroxyl groups. They are synthesised mainly through the shikimate and phenylpropanoid pathways, and more than 8,000 phenolic structures have been identified in plants, ranging from simple phenolic acids to highly polymerised tannins *(Balasundram et al., 2006; Pandey & Rizvi, 2009)*. The major classes of plant phenolics are summarised in Table 1.1.

Class	Basic skeleton	Examples
Hydroxybenzoic acids	C_6–C_1	Gallic acid, protocatechuic acid, vanillic acid, ellagic acid
Hydroxycinnamic acids	C_6–C_3	Caffeic acid, ferulic acid, p-coumaric acid, chlorogenic acid
Flavonoids	C_6–C_3–C_6	Quercetin, kaempferol, catechin, apigenin, naringenin
Stilbenes	C_6–C_2–C_6	Resveratrol
Lignans	$(C_6$–$C_3)_2$	Secoisolariciresinol, matairesinol
Tannins	Polymeric	Gallotannins, ellagitannins (hydrolysable); proanthocyanidins (condensed)
Coumarins	C_6–C_3 (lactone)	Umbelliferone, scopoletin

Table 1.1: Major classes of plant phenolic compounds *(Balasundram et al., 2006; Dai & Mumper, 2010; Tungmunnithum et al., 2018)*



(a) Phenol (basic phenolic unit) **(b) Gallic acid (3,4,5-trihydroxybenzoic acid)**

Figure 1.2: Structures of (a) phenol, the basic phenolic unit, and (b) gallic acid, the standard used in the total phenolic content assay.

1.4.2 Mechanism of Antioxidant Action of Phenolics

Phenolic compounds act as antioxidants mainly because their hydroxyl groups can donate a hydrogen atom or an electron to free radicals, and the resulting phenoxyl radical is stabilised by delocalisation of the unpaired electron over the aromatic ring *(Rice-Evans et al., 1996)*. Three principal mechanisms have been described *(Leopoldini et al., 2011; Gulcin, 2020)*:

- **Hydrogen atom transfer (HAT):** the phenol donates a hydrogen atom directly to the radical ($ArOH + R^{\bullet} \rightarrow ArO^{\bullet} + RH$).
- **Single electron transfer followed by proton transfer (SET-PT):** an electron is first transferred to the radical, forming a radical cation, which then loses a proton ($ArOH + R^{\bullet} \rightarrow ArOH^{\bullet+} + R^{-} \rightarrow ArO^{\bullet} + RH$).

- **Sequential proton loss electron transfer (SPLET):** the phenol first dissociates into a phenoxide anion, which then transfers an electron to the radical; this pathway is favoured in polar solvents.
- **Metal chelation:** compounds with catechol or galloyl groups bind transition metal ions such as Fe^{2+} and Cu^{2+}, preventing them from catalysing the formation of the hydroxyl radical through Fenton-type reactions.

1.4.3 Structural Features Influencing Antioxidant Activity

The antioxidant activity of phenolic compounds depends strongly on their structure. In general, activity increases with the number of hydroxyl groups; an ortho-dihydroxy (catechol) or 3,4,5-trihydroxy (galloyl) arrangement markedly enhances radical-scavenging ability because it stabilises the phenoxyl radical by intramolecular hydrogen bonding and electron delocalisation. For flavonoids, the key features are a catechol group in ring B, a 2,3-double bond in conjugation with a 4-oxo group in ring C, and hydroxyl groups at positions 3 and 5. Glycosylation of hydroxyl groups generally reduces activity compared with the corresponding aglycone *(Rice-Evans et al., 1996; Pietta, 2000; Heim et al., 2002)*. Quercetin, which fulfils all of these criteria, is therefore one of the most potent natural antioxidants.

1.5 Flavonoids

1.5.1 Structure and Classification

Flavonoids are the largest group of plant phenolics, with several thousand compounds described. Their basic structure is a fifteen-carbon (C_6–C_3–C_6) skeleton consisting of two aromatic rings (A and B) linked by a three-carbon bridge that usually forms an oxygen-containing heterocyclic ring (C) *(Kumar & Pandey, 2013; Panche et al., 2016)*. Depending on the oxidation level and substitution pattern of ring C, flavonoids are divided into several subclasses (Table 1.2).

Subclass	Characteristic structural feature	Representative compounds
Flavones	2,3-double bond and 4-oxo group in ring C	Apigenin, luteolin
Flavonols	Flavone with a 3-hydroxyl group	Quercetin, kaempferol, myricetin
Flavanones	Saturated C2–C3 bond, 4-oxo group	Naringenin, hesperetin
Flavan-3-ols (flavanols)	No 4-oxo group, 3-hydroxyl group	Catechin, epicatechin, epigallocatechin gallate
Isoflavones	Ring B attached at C-3	Genistein, daidzein
Anthocyanidins	Flavylium cation	Cyanidin, delphinidin, pelargonidin
Chalcones	Open ring C	Phloretin, butein

Table 1.2: Major subclasses of flavonoids and representative compounds *(Kumar & Pandey, 2013; Panche et al., 2016)*

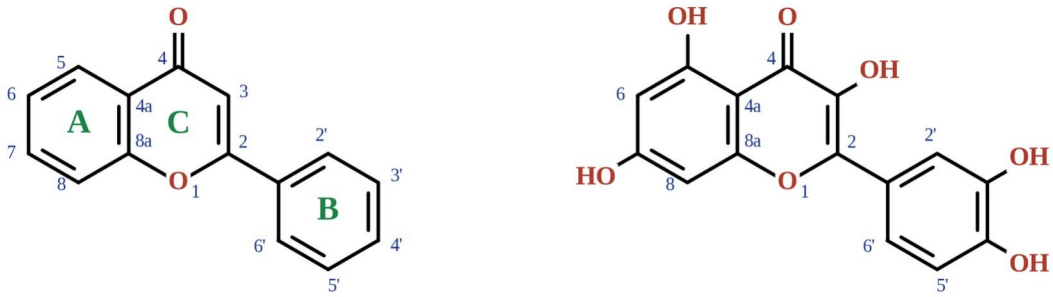


Figure 1.3: (a) Basic flavone nucleus showing rings A, B and C with carbon numbering; (b) structure of quercetin, the standard used in the total flavonoid content assay and a major flavonol of guava leaves.

1.5.2 Role in Plant Defence and Health Benefits

In plants, flavonoids contribute to flower and fruit pigmentation, protect tissues against ultraviolet radiation, act as signalling molecules and participate in defence against microorganisms and herbivores *(Panche et al., 2016; Tungmunnithum et al., 2018)*. In humans, dietary flavonoids have been associated with antioxidant, anti-inflammatory, antimicrobial, antiviral, anticancer, cardioprotective, hepatoprotective and antidiabetic activities *(Kumar & Pandey, 2013; Panche et al., 2016)*. Guava leaves are particularly rich in flavonols, especially quercetin and its glycosides such as guaijaverin, avicularin, isoquercitrin and hyperoside, together with flavan-3-ols such as catechin and epicatechin *(Díaz-de-Cerio et al., 2017; Kumar et al., 2021)*.

1.6 Antioxidant Assays

Because plant extracts contain complex mixtures of compounds that act through different mechanisms, no single method can fully describe their antioxidant capacity. It is therefore recommended to use a combination of complementary assays *(Prior et al., 2005; Munteanu & Apetrei, 2021)*. In this study, the DPPH radical scavenging assay was used to measure antioxidant activity, while the TPC and TFC assays were used to quantify the phenolic and flavonoid constituents that may be responsible for this activity. The principles of the three assays are summarised in Table 1.3 and described below.

Assay	Principle	Wavelength	Standard	Expression of result
DPPH radical scavenging	Reduction of the purple $DPPH^{\bullet}$ radical by H-atom/electron donation	517 nm	Ascorbic acid	% inhibition; IC_{50} (µg/mL)
Total phenolic content (Folin–Ciocalteu)	Reduction of phosphomolybdate–phosphotungstate reagent to a blue complex in alkaline medium	765 nm	Gallic acid	mg GAE/g extract
Total flavonoid content ($AlCl_3$)	Formation of a yellow Al^{3+}–flavonoid complex	415 nm	Quercetin	mg QE/g extract

Table 1.3: Summary of the assays used in the present study

1.6.1 DPPH Radical Scavenging Assay

The DPPH method was first described by *Blois (1958)* and later modified by *Brand-Williams et al. (1995)*; it is now one of the most widely used methods for screening the antioxidant activity of plant extracts. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable, nitrogen-centred free radical that is deep violet in methanolic solution and shows a strong absorption maximum at about 515–517 nm. When it accepts a hydrogen atom or an electron from an antioxidant, it is reduced to the non-radical hydrazine form (DPPH–H), and the colour changes from violet to pale yellow. The resulting decrease in absorbance is proportional to the radical-scavenging capacity of the sample *(Molyneux, 2004; Kedare & Singh, 2011)*.

Advantages: the assay is simple, rapid, inexpensive and reproducible; it uses a commercially available stable radical that does not need to be generated, and requires only a UV–Visible spectrophotometer *(Kedare & Singh, 2011)*. **Limitations:** DPPH is not a physiological radical; its bulky structure means that small molecules have better access to the radical site than large molecules such as tannins; compounds that absorb at

517 nm (e.g., coloured pigments) can interfere; and the results depend on the solvent, pH, reaction time and DPPH concentration, which makes inter-laboratory comparisons difficult *(Prior et al., 2005; Kedare & Singh, 2011; Munteanu & Apetrei, 2021)*.

1.6.2 Total Phenolic Content (Folin–Ciocalteu) Assay

The Folin–Ciocalteu (FC) method, standardised by *Singleton and Rossi (1965)*, is the most common method for estimating total phenolics. The FC reagent is a mixture of phosphomolybdic and phosphotungstic acids. Under alkaline conditions (provided by sodium carbonate), phenolic compounds dissociate to phenolate anions, which reduce the molybdenum(VI) centres of the reagent to molybdenum(V), producing a blue-coloured complex with maximum absorbance at about 750–765 nm *(Singleton et al., 1999; Ainsworth & Gillespie, 2007)*. The results are expressed as gallic acid equivalents (GAE). Although simple and reproducible, the assay actually measures total reducing capacity, and non-phenolic reducing substances such as ascorbic acid, reducing sugars and some amino acids and proteins can also react with the reagent *(Everette et al., 2010; Sánchez-Rangel et al., 2013)*.

1.6.3 Total Flavonoid Content (Aluminium Chloride) Assay

The aluminium chloride colorimetric method is widely used to estimate total flavonoids. Aluminium(III) ions form stable complexes with the C-4 keto group and the C-3 or C-5 hydroxyl group of flavones and flavonols, and more labile complexes with ortho-dihydroxyl groups in ring A or B. Complex formation causes a bathochromic shift and an intensification of the yellow colour, which is measured at about 415 nm when quercetin is used as the standard; potassium acetate is added to stabilise the complex *(Chang et al., 2002; Pękal & Pyrzynska, 2014)*. The response of individual flavonoids differs according to their structure, and flavanones and flavan-3-ols react weakly, so the results depend on the reference standard and should be interpreted as estimates of the flavone/flavonol content *(Pękal & Pyrzynska, 2014; Shraim et al., 2021)*.

1.7 Factors Affecting Phenolic Content and Antioxidant Activity

The phenolic content and antioxidant activity of a plant extract are influenced by several pre-extraction and extraction-related factors:

- **Solvent polarity:** phenolic compounds differ widely in polarity. Aqueous–organic mixtures, such as 50–70% ethanol or methanol, are often more efficient than pure solvents *(Sultana et al., 2009; Do et al., 2014)*. For guava leaves, 50%

hydroethanolic extraction gave the highest phenolic content and antioxidant activity *(Seo et al., 2014)*, and phenolic yield decreased at ethanol concentrations above about 55% *(Dang Thi et al., 2024)*.

- **Extraction method and time:** for guava leaves, ultrasound-assisted extraction gave extracts with higher antioxidant activity than maceration with agitation, maceration without agitation or Soxhlet extraction *(Nantitanon et al., 2010)*. Extraction time must be long enough for diffusion but not so long that compounds degrade *(Azwanida, 2015)*.
- **Temperature:** moderate heating improves solubility and diffusion, but excessive heat degrades thermolabile phenolics; therefore low-temperature methods such as cold maceration and evaporation under reduced pressure are preferred *(Dai & Mumper, 2010; Azwanida, 2015)*.
- **Drying and particle size:** shade drying protects light- and heat-sensitive constituents, and grinding into a powder increases the surface area available for solvent contact *(Abubakar & Haque, 2020)*.
- **Plant-related factors:** cultivar, leaf maturity, season of harvest, geographical origin and growing conditions strongly influence phenolic composition *(Kumar et al., 2021)*. Different guava varieties, for example, have shown markedly different phenolic contents and radical-scavenging activities *(Camarena-Tello et al., 2018; Sowmya & Usha Anandhi, 2020)*.

1.8 Correlation Between Phenolic Content and Antioxidant Activity

Numerous studies have reported that plants with a higher phenolic content tend to show stronger antioxidant activity, because phenolics are the main hydrogen- and electron-donating constituents of most plant extracts *(Balasundram et al., 2006; Dai & Mumper, 2010)*. For guava leaves, *Seo et al. (2014)* found that antioxidant ability was more strongly related to phenolic content than to flavonoid content, and *Hartati et al. (2020)* reported a significant positive correlation between the TPC of guava leaf extracts and their DPPH antioxidant activity index. However, such correlations are not universal, because the activity of an extract depends on the types of phenolics present, their interactions and the specific assay conditions *(Hatami et al., 2014)*. Measuring both the phenolic/flavonoid content and the radical-scavenging activity of the same extract, as done in this study, therefore provides a more complete picture of its antioxidant potential.

1.9 Rationale of the Research

Although many studies have evaluated the antioxidant properties of guava leaves, the reported values vary widely because of differences in plant variety, geographical origin, season, extraction solvent and method, and assay conditions *(Nantitanon et al., 2010; Seo et al., 2014; Kumar et al., 2021)*. Data on guava leaves grown in Bangladesh and extracted by simple cold maceration with ethanol, a relatively safe and food-compatible solvent, remain limited. Since guava leaves are abundant and inexpensive and have long been used in traditional medicine, a systematic evaluation of their antioxidant activity together with their phenolic and flavonoid contents can provide useful preliminary scientific evidence for their therapeutic potential and future development as natural antioxidant products.

1.10 Aim and Objectives of the Study

General objective: to evaluate the *in vitro* antioxidant potential of the ethanolic leaf extract of *Psidium guajava* L. and to quantify its total phenolic and total flavonoid contents.

Specific objectives:

1. To prepare a crude ethanolic extract from shade-dried leaves of *P. guajava* by cold maceration.
2. To evaluate the free radical scavenging activity of the extract using the DPPH assay and to determine its IC_{50} value in comparison with the standard antioxidant ascorbic acid.
3. To determine the total phenolic content of the extract by the Folin–Ciocalteu method, expressed as mg gallic acid equivalents per gram of extract (mg GAE/g).
4. To determine the total flavonoid content of the extract by the aluminium chloride method, expressed as mg quercetin equivalents per gram of extract (mg QE/g).
5. To examine the relationship between the phenolic and flavonoid contents and the radical-scavenging activity of the extract, and to compare the findings with previously published studies.

1.11 Significance of the Study

The findings of this study are expected to (i) provide scientific evidence supporting the traditional medicinal use of guava leaves; (ii) generate baseline data on the antioxidant activity and phenolic/flavonoid composition of guava leaves collected in Bangladesh; (iii) highlight the potential of an abundant and inexpensive agricultural by-product as a source of natural antioxidants for functional foods, nutraceuticals, cosmetics and pharmaceutical formulations; and (iv) serve as a foundation for further phytochemical, cell-based and *in vivo* studies on the therapeutic potential of *P. guajava* leaves in oxidative stress-related diseases.

CHAPTER 2
PLANT PROFILE

2.1 Plant Family: Myrtaceae

The Myrtaceae, or myrtle family, belongs to the order Myrtales and is one of the largest families of flowering plants, comprising more than 130 genera and several thousand species of evergreen trees and shrubs *(Christenhusz & Byng, 2016)*. Members of the family are distributed mainly in tropical and warm-temperate regions, with centres of diversity in Australia, South-East Asia and tropical America. Characteristic features of the family include simple, opposite, entire leaves with translucent oil glands that make the foliage aromatic, flowers with numerous conspicuous stamens, and fruits that are berries or capsules. The family contains many economically important plants, such as *Eucalyptus* species (timber and eucalyptus oil), clove (*Syzygium aromaticum*), black plum or *kalo jam* (*Syzygium cumini*), rose apple (*Syzygium jambos*), allspice (*Pimenta dioica*), tea tree (*Melaleuca alternifolia*) and guava (*Psidium guajava*).

2.2 Plant Genus: *Psidium*

The genus *Psidium* L. is native to tropical and subtropical America, extending from Mexico and the Caribbean to Argentina and Uruguay, with its greatest diversity in eastern Brazil. Estimates of the number of species vary with the taxonomic treatment; the most recent monograph of the genus recognises 63 species *(Landrum, 2025)*. *Psidium* species are shrubs or trees, often with strongly pinnate-veined, gland-dotted leaves, white flowers with numerous stamens, and many-seeded berries crowned by persistent calyx lobes. Besides the common guava (*P. guajava*), other species such as the strawberry or cherry guava (*P. cattleyanum*) and the Brazilian guava (*P. guineense*) are cultivated for their edible fruits.

2.3 Plant Species: *Psidium guajava* L.

Psidium guajava L. is the most important and widely cultivated species of the genus. Native to tropical America, it has become naturalised and cultivated throughout the tropics and subtropics, and it is an important fruit crop in countries such as India, Indonesia, Pakistan, Bangladesh, Mexico and Brazil *(Gutiérrez et al., 2008; Kumar et al., 2021)*. Almost all parts of the plant, including the leaves, fruits, bark and roots, have been used in traditional medicine, but the leaves are the most extensively studied part *(Díaz-de-Cerio et al., 2017)*.

2.4 Taxonomic Hierarchy

Taxonomic rank	Name
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Myrtales
Family	Myrtaceae
Genus	*Psidium* L.
Species	*Psidium guajava* L.

Table 2.1: Taxonomic classification of *Psidium guajava* L.

2.5 Scientific Name and Synonyms

Scientific name: *Psidium guajava* L. (Linnaeus, 1753).

Synonyms: *Psidium pyriferum* L. and *Psidium pomiferum* L., names that Linnaeus originally used for the pear-shaped and apple-shaped forms of the fruit, respectively, and *Guajava pyrifera* (L.) Kuntze *(Gutiérrez et al., 2008)*.

2.6 Common and Vernacular Names

Language / Region	Common name
English	Guava, common guava, apple guava
Bengali (Bangla)	Peyara ( )
Hindi / Urdu	Amrood, amrud
Tamil	Koyya
Spanish	Guayaba
Portuguese	Goiaba
French	Goyave
Indonesian / Malay	Jambu biji
Thai	Farang
Filipino	Bayabas
Chinese	Fan shi liu

Table 2.2: Common and vernacular names of *Psidium guajava* L. *(Gutiérrez et al., 2008; Kumar et al., 2021)*

2.7 Distribution and Habitat

Guava is thought to have originated in the region extending from southern Mexico to Central America and northern South America. It was spread by early traders and explorers and is now grown in almost all tropical and subtropical countries *(Gutiérrez et al., 2008)*. The plant is hardy and adaptable: it grows in a wide range of soils, from sandy to clay, tolerates drought and moderate salinity, and thrives in warm, humid climates, although it can survive brief cool periods. In Bangladesh, guava is grown in homesteads and commercial orchards throughout the country, and it is one of the most popular and affordable seasonal fruits. Because the trees are evergreen and are pruned regularly, large quantities of leaves are available year-round, most of which are discarded.

2.8 Botanical Description

P. guajava is a small evergreen tree or large shrub, usually 3–10 m tall, with a spreading crown *(Gutiérrez et al., 2008; Naseer et al., 2018)*. Its main morphological features are:

- **Stem and bark:** the trunk is often crooked and branched close to the ground; the bark is thin, smooth and greenish-brown to reddish-brown, and it peels off in thin flakes, leaving a mottled surface. Young twigs are quadrangular and finely pubescent.
- **Leaves:** simple, opposite and short-petiolate; oval to oblong-elliptic, about 5–15 cm long and 3–7 cm wide; somewhat leathery, with an entire margin, a blunt or slightly pointed apex and conspicuous, parallel pinnate veins that are impressed on the upper surface and prominent beneath. The lower surface is more or less downy, and the leaves are aromatic when crushed because of their essential-oil glands *(Gutiérrez et al., 2008; Kumar et al., 2021)*.
- **Flowers:** white, fragrant, about 2.5–3 cm in diameter, borne singly or in small clusters of two or three in the leaf axils, with four to six petals and numerous stamens with yellow anthers.
- **Fruit:** a globose, ovoid or pear-shaped berry, usually 3–10 cm long, crowned by the persistent calyx lobes. The skin is green when immature and turns yellowish when ripe; the pulp is white, pink or red, sweet to slightly acidic, and aromatic.
- **Seeds:** numerous, small, hard and kidney-shaped, embedded in the central pulp.



Figure 2.1: Shade-dried leaves of *Psidium guajava* L. used in the present study.

2.9 Traditional Uses

Guava leaves have been used in folk medicine in Latin America, Africa and Asia for centuries. A decoction or infusion of the leaves is the best known remedy, and the leaves are also chewed or applied externally as a poultice *(Gutiérrez et al., 2008; Morais-Braga et al., 2016; Naseer et al., 2018)*. The principal traditional uses of the leaves include the management of:

- Diarrhoea, dysentery and gastroenteritis (the most common traditional use)
- Stomach ache, indigestion and flatulence
- Diabetes mellitus (guava leaf tea is widely consumed in East Asia)
- Cough, sore throat and other respiratory complaints
- Toothache, swollen gums, mouth ulcers and bad breath (leaves are chewed or used as a mouthwash)
- Wounds, ulcers, boils and skin infections (applied externally)
- Fever and malaria

- Vaginal discharge (leucorrhoea) and menstrual pain
- Rheumatic pain and conjunctivitis

In Bangladesh and the wider Indian subcontinent, tender guava leaves are commonly chewed to relieve toothache and mouth ulcers, and a leaf decoction is taken for diarrhoea. The extensive traditional use of guava leaves has encouraged numerous scientific investigations into their chemical composition and pharmacological properties *(Díaz-de-Cerio et al., 2017).*

2.10 Phytochemical Constituents of Guava Leaves

Phytochemical investigations have shown that guava leaves contain a complex mixture of primary and secondary metabolites. Qualitative screening consistently reveals phenols, flavonoids, tannins, terpenoids, saponins, glycosides and steroids *(Möwes et al., 2025)*, and detailed chromatographic studies have identified a large number of individual compounds (Table 2.3). Polyphenols, particularly flavonol glycosides, flavan-3-ols, phenolic acids and ellagitannins, are considered the principal antioxidant constituents *(Díaz-de-Cerio et al., 2017; Kumar et al., 2021)*.

Class of compounds	Representative constituents
Flavonols and their glycosides	Quercetin, guaijaverin (quercetin-3-O-arabinopyranoside), avicularin (quercetin-3-O-arabinofuranoside), isoquercitrin, hyperoside (hyperin), quercitrin, reynoutrin, kaempferol, myricetin, morin glycosides
Other flavonoids	Apigenin, luteolin, catechin, epicatechin, gallocatechin, epigallocatechin gallate, proanthocyanidins
Phenolic acids	Gallic acid, ellagic acid, chlorogenic acid, caffeic acid, ferulic acid, protocatechuic acid
Tannins	Ellagitannins (e.g., pedunculagin, vescalagin, castalagin) and gallotannins
Benzophenones	Benzophenone glycosides (e.g., guavinosides)
Triterpenoids and sterols	Ursolic acid, oleanolic acid, asiatic acid, corosolic acid, maslinic acid, guajanoic acid, uvaol, β-sitosterol
Meroterpenoids	Guajadial, psidials and related sesquiterpene-based meroterpenoids
Essential oil components	β-Caryophyllene (major), caryophyllene oxide, α-humulene, limonene, 1,8-cineole, α-pinene, nerolidol, β-bisabolene
Others	Carotenoids, chlorophylls, ascorbic acid, polysaccharides, saponins, proteins and minerals

Table 2.3: Major phytochemical constituents reported in guava leaves *(Gutiérrez et al., 2008; Díaz-de-Cerio et al., 2017; Naseer et al., 2018; Kumar et al., 2021)*

The composition of guava leaves varies with the variety, leaf age, season, geographical location and drying method, which partly explains the variability in the biological activities reported by different studies *(Kumar et al., 2021; Dang Thi et al., 2024)*.

2.11 Pharmacological Activities of Guava Leaves

Extracts and isolated constituents of guava leaves have been investigated in a wide range of *in vitro*, animal and clinical studies. Pharmacological studies have demonstrated antioxidant, hepatoprotective, antiallergic, antimicrobial, antigenotoxic, antiplasmodial, cytotoxic, antispasmodic, cardioactive, anticough, antidiabetic, anti-inflammatory and antinociceptive activities, many of which support the traditional uses of the plant *(Gutiérrez et al., 2008; Sanda et al., 2011; Shruthi et al., 2013)*. The most important activities are summarised below.

2.11.1 Antioxidant Activity

Guava leaves are among the most extensively studied plant materials with respect to antioxidant activity. Extracts prepared with water, ethanol, methanol, acetone and their mixtures have shown dose-dependent scavenging of DPPH, ABTS, superoxide, hydroxyl and nitric oxide radicals, reducing power and inhibition of lipid peroxidation *(Qian & Nihorimbere, 2004; Chen & Yen, 2007; Seo et al., 2014)*. *Tachakittirungrod et al. (2007)* found guava leaf extract to be among the most active of several Thai plants tested and attributed its effect largely to the radical-scavenging action of its phenolic constituents. The high antioxidant activity of guava leaves is generally attributed to their polyphenols, especially quercetin glycosides, catechins, phenolic acids and tannins *(Díaz-de-Cerio et al., 2017; Kumar et al., 2021)*.

2.11.2 Antidiabetic Activity

Guava leaf extract lowered blood glucose in type 2 diabetic $Lepr^{db}/Lepr^{db}$ mice, an effect partly mediated by inhibition of protein tyrosine phosphatase 1B *(Oh et al., 2005)*. The anti-hyperglycaemic and anti-hyperlipidaemic effects of guava leaf extract have been reviewed by *Deguchi and Miyazaki (2010)*, who noted that guava leaf tea has been approved in Japan as a food for specified health uses for people with borderline or mild hyperglycaemia. Aqueous guava leaf extract also strongly inhibited the carbohydrate-digesting enzyme α-glucosidase *in vitro*, with a lower IC_{50} than the drug acarbose *(Simamora et al., 2018)*.

2.11.3 Antidiarrhoeal and Antispasmodic Activity

The use of guava leaves for diarrhoea is supported by experimental and clinical evidence. In a clinical study, a phytodrug prepared from guava leaves (standardised to quercetin) reduced the duration of abdominal pain in adults with acute diarrhoeal disease, an effect attributed to its intestinal antispasmodic activity *(Lozoya et al., 2002; Gutiérrez et al., 2008)*.

2.11.4 Antimicrobial Activity

Guava leaf extracts inhibit the growth of a range of Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Salmonella* species *(Biswas et al., 2013)*. Antimicrobial flavonoid glycosides and other phenolic compounds have been isolated from guava *(Arima & Danno, 2002; Metwally et al., 2010)*, and the ethnobotanical use of the plant against infections has been reviewed in detail *(Morais-Braga et al., 2016)*.

2.11.5 Anti-inflammatory and Analgesic Activity

An aqueous leaf extract of *P. guajava* produced significant anti-inflammatory and analgesic effects in rats and mice *(Ojewole, 2006)*. Similarly, an ethanolic leaf extract (250 and 500 mg/kg) significantly inhibited acute (carrageenan-induced paw oedema), subacute (granuloma pouch) and chronic (Freund's adjuvant-induced arthritis) inflammation in rats *(Dutta & Das, 2010)*.

2.11.6 Anticancer and Cytotoxic Activity

Guava leaf extracts and their constituents have shown antiproliferative and cytotoxic effects against several cancer cell lines, and essential-oil and extract fractions have demonstrated antitumour and anticancer potential *(Ashraf et al., 2016)*. A network pharmacology study identified multiple guava leaf constituents and targets that may contribute to activity against lung cancer *(Jiang et al., 2020)*. These effects are thought to involve antioxidant, anti-inflammatory and pro-apoptotic mechanisms, although most of the evidence is preclinical *(Kumar et al., 2021)*.

2.11.7 Hepatoprotective, Immunomodulatory and Other Activities

Guava leaf extract protected rats against chemically induced liver damage *(Roy et al., 2006)*, and guava leaves have been proposed as a functional immunostimulatory ingredient *(Laily et al., 2015)*. Other reported activities include antiallergic, antitussive,

antigenotoxic, antiplasmodial and cardioprotective effects *(Gutiérrez et al., 2008; Díaz-de-Cerio et al., 2017)*. The main activities are summarised in Table 2.4.

Activity	Key finding	Reference
Antioxidant	Dose-dependent scavenging of DPPH and other radicals; high phenolic content	*Qian & Nihorimbere (2004)*; *Chen & Yen (2007)*
Antidiabetic	Lowered blood glucose in diabetic mice; PTP1B inhibition	*Oh et al. (2005)*
Antidiabetic	Potent α-glucosidase inhibition (IC_{50} 5.67 µg/mL)	*Simamora et al. (2018)*
Antidiarrhoeal	Reduced abdominal pain in acute diarrhoea (clinical study)	*Lozoya et al. (2002)*
Antimicrobial	Inhibition of Gram-positive and Gram-negative bacteria	*Biswas et al. (2013)*
Anti-inflammatory	Inhibition of acute, subacute and chronic inflammation in rats	*Dutta & Das (2010)*
Analgesic	Antinociceptive effect in mice and rats	*Ojewole (2006)*
Anticancer	Cytotoxic and antitumour effects of leaf extracts	*Ashraf et al. (2016)*
Hepatoprotective	Protection against chemically induced liver injury	*Roy et al. (2006)*
Immunostimulatory	Enhancement of immune response	*Laily et al. (2015)*

Table 2.4: Summary of selected pharmacological activities of guava leaf extracts

2.12 Previous Studies on the Antioxidant Activity of Guava Leaves and Research Gap

Several studies have quantified the phenolic content and radical-scavenging activity of guava leaf extracts. *Qian and Nihorimbere (2004)* reported remarkably high total phenolic contents in 50% ethanol and water extracts of guava leaves and found ascorbic acid to be a more powerful antioxidant than the extracts. *Seo et al. (2014)* compared water, ethanol, methanol and hydroethanolic solvents and found 50% ethanol to be the most effective. *You et al. (2011)* reported that among extracts of different parts of the guava tree, the leaf and branch extracts had the highest antioxidant activity. Studies from India, Indonesia, Mexico, Brazil and Vietnam have reported DPPH IC_{50} values ranging from below 10 µg/mL to more than 250 µg/mL, depending on the variety, solvent, extraction method and assay conditions *(Fernandes et al., 2014; Camarena-Tello et al., 2018; Simamora et al., 2018; Sowmya & Usha Anandhi, 2020; Dang Thi et al., 2024)*. These studies are compared with the results of the present work in Chapter 5 (Table 5.1).

Despite this body of work, relatively few studies have simultaneously evaluated the DPPH radical-scavenging activity, total phenolic content and total flavonoid content of

guava leaves collected in Bangladesh using simple cold maceration with ethanol. The present study was designed to address this gap and to generate preliminary data that can support further pharmacological research on this abundant local resource.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Design

The present work was an experimental, laboratory-based *in vitro* study designed to evaluate the antioxidant potential of the ethanolic leaf extract of *Psidium guajava* L. The free radical scavenging activity of the extract was determined by the DPPH assay using ascorbic acid as the reference standard. The total phenolic content was determined by the Folin–Ciocalteu method using gallic acid as the standard, and the total flavonoid content was determined by the aluminium chloride colorimetric method using quercetin as the standard. The overall workflow of the study is shown in Figure 3.1.

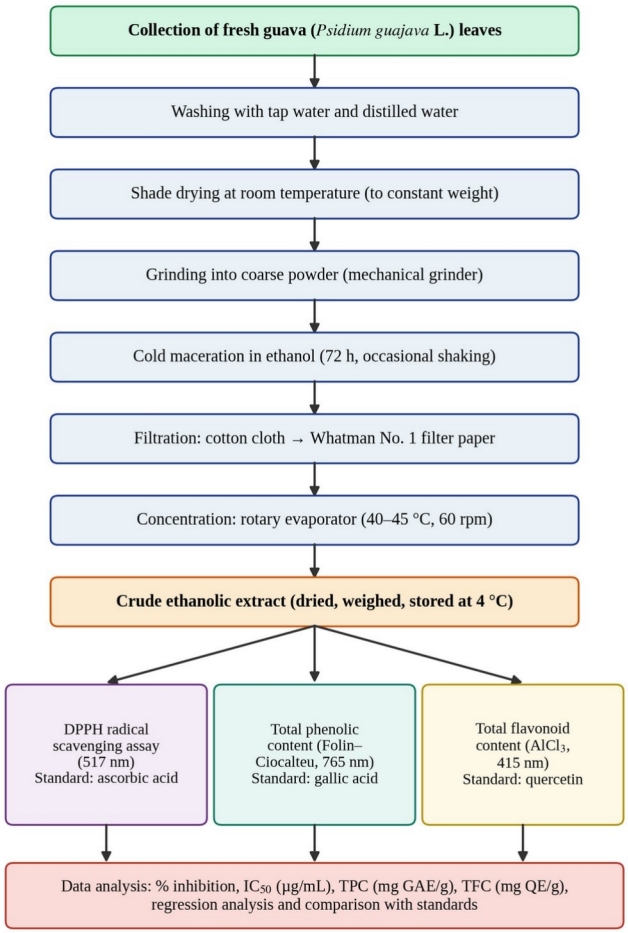


Figure 3.1: Schematic flowchart of the overall study design.

3.2 Study Area

All experimental work was carried out in the Pharmaceutical Sciences Laboratory, Department of Pharmacy, Independent University, Bangladesh (IUB), Bashundhara, Dhaka, Bangladesh.

3.3 Collection and Identification of Plant Material

Fresh, mature and healthy leaves of *Psidium guajava* L. were plucked by hand directly from the tree in Birulia, Savar, Dhaka, Bangladesh, on 27 February 2026. Approximately 3 kg of fresh leaves was collected. Leaves that were free from disease, insect damage and mechanical injury were selected. The plant was identified on the basis of its characteristic morphological features (Section 2.8) with reference to standard descriptions *(Gutiérrez et al., 2008)*. The collected leaves were transported to the laboratory in clean polyethylene bags for further processing.

3.4 Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade. The chemicals and their purposes are listed in Table 3.1.

SL	Chemical / reagent	Purpose
1	Ethanol (98%)	Extraction solvent
2	Methanol	Solvent for DPPH, standards and extract solutions; blank
3	DPPH (2,2-diphenyl-1-picrylhydrazyl)	Stable free radical for the antioxidant assay
4	Ascorbic acid	Standard antioxidant (positive control) for the DPPH assay
5	Folin–Ciocalteu reagent	Chromogenic reagent for total phenolic content
6	Sodium carbonate (Na_2CO_3)	Provides the alkaline medium in the TPC assay
7	Gallic acid	Standard for the TPC calibration curve
8	Aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O$)	Complexing agent in the TFC assay
9	Potassium acetate (CH_3COOK)	Stabiliser of the Al^{3+}–flavonoid complex
10	Quercetin	Standard for the TFC calibration curve
11	Distilled water	Washing, reagent preparation and dilution

Table 3.1: Chemicals and reagents used in the study

3.5 Equipment and Apparatus

SL	Instrument / apparatus	Purpose
1	Rotary vacuum evaporator (LabTech)	Concentration of the ethanolic filtrate under reduced pressure
2	UV–Visible spectrophotometer	Absorbance measurements at 517, 765 and 415 nm
3	Analytical balance (ISOLAB)	Accurate weighing of extract, standards and reagents
4	Mechanical grinder	Grinding of dried leaves
5	Micropipettes and pipettes	Accurate transfer of small volumes
6	Vortex mixer	Dissolution and mixing of solutions
7	Refrigerator (4 °C)	Storage of the crude extract
8	Fume hood	Evaporation of residual solvent from the extract
9	Amber reagent bottle and amber volumetric flasks	Maceration and light-protected preparation of solutions
10	Test tubes, test tube rack, beakers, measuring cylinders, funnels	General laboratory work
11	Whatman No. 1 filter paper and clean cotton cloth	Filtration of the macerate
12	Petri dishes, 15 mL tubes, aluminium foil, spatula	Drying, collection and storage of the extract

Table 3.2: Instruments and apparatus used in the study

3.6 Preparation of the Plant Extract

3.6.1 Cleaning of Plant Material

The freshly collected leaves were washed thoroughly under running tap water to remove dust, soil particles and other impurities, rinsed with distilled water, and spread on clean paper to remove excess water.

3.6.2 Drying

The cleaned leaves were shade-dried at room temperature, with occasional turning, until they became crisp and reached a constant weight (Figure 2.1). Approximately 3 kg of fresh leaves gave about 900 g of dried leaves, which corresponds to a loss of about 70% of the fresh weight as moisture during drying. Direct sunlight was avoided to prevent the degradation of light- and heat-sensitive phytochemicals such as phenolic compounds and flavonoids *(Abubakar & Haque, 2020)*.

3.6.3 Grinding

The dried leaves were ground into a coarse powder using a mechanical grinder. Size reduction increases the surface area of the plant material and improves contact with the extraction solvent *(Azwanida, 2015)*. The powder was stored in a clean, dry, airtight container until extraction.

3.6.4 Ethanolic Extraction (Cold Maceration)

A clean, dry amber-coloured reagent bottle was used for extraction. From the dried material, 400 g of the coarsely powdered leaves was placed in the bottle, and 800 mL of 98% ethanol was added (powder-to-solvent ratio 1:2, w/v); the contents were mixed thoroughly so that the powder was uniformly wetted by the solvent. The mouth of the bottle was sealed with aluminium foil to minimise solvent evaporation, and the mixture was macerated at room temperature for 3 days (72 hours) with occasional shaking to facilitate the extraction of the constituents.

Ethanol was selected as the extraction solvent because it efficiently dissolves a broad range of polar and moderately polar compounds, including phenolic acids, flavonoids and their glycosides, and because it is relatively safe, food-compatible and easy to remove by evaporation *(Azwanida, 2015; Zhang et al., 2018)*. Cold maceration was chosen because it is simple and avoids heating, thereby protecting thermolabile compounds *(Abubakar & Haque, 2020)*.

3.6.5 Filtration

At the end of the maceration period, the mixture was first filtered through a clean cotton cloth to remove coarse plant debris, and the filtrate was then passed through Whatman No. 1 filter paper to obtain a clear ethanolic extract.

3.6.6 Concentration of the Extract

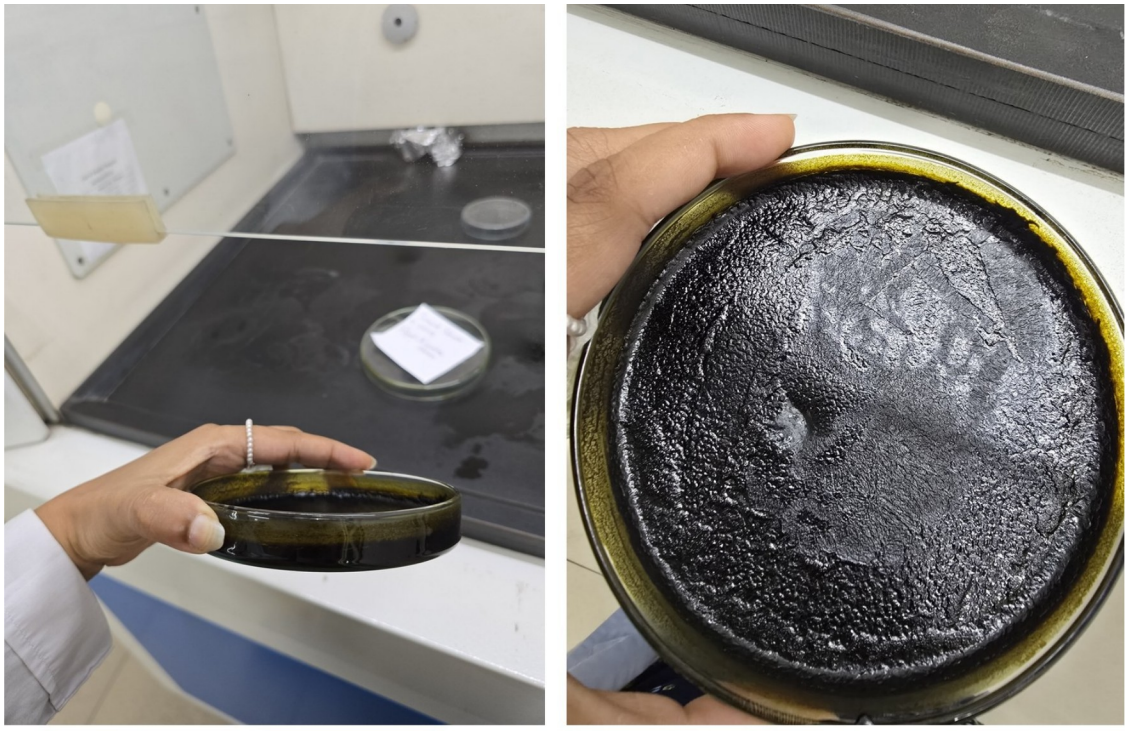
The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator (Figure 3.2) to remove the ethanol. The operating conditions were: water-bath temperature 40–45 °C, condenser (cooling) temperature 5 °C and rotation speed 60 rpm. Evaporation under reduced pressure at a low temperature minimises the thermal degradation of phenolic compounds *(Dai & Mumper, 2010)*.



Figure 3.2: Concentration of the ethanolic leaf extract of *P. guajava* using a rotary vacuum evaporator.

3.6.7 Drying and Collection of the Crude Extract

The concentrated extract was transferred to clean glass Petri dishes and kept in a fume hood at room temperature to allow the residual solvent to evaporate completely. The dried crude extract was a dark green to blackish, sticky, semi-solid mass with a greenish-yellow margin (Figure 3.3), a colour typical of leaf extracts that contain chlorophyll together with polyphenols.



(a) **(b)**

Figure 3.3: Crude ethanolic extract of *P. guajava* leaves after rotary evaporation and drying in a Petri dish: (a) side view and (b) top view.

3.6.8 Weighing and Storage of the Crude Extract

The dried crude extract was collected, weighed accurately using an analytical balance (Figure 3.4) and transferred into a clean, labelled, airtight container. The extract was stored in a refrigerator at 4 °C, protected from light, until it was used for the DPPH, TPC and TFC assays, in order to minimise oxidative degradation of its phenolic constituents.

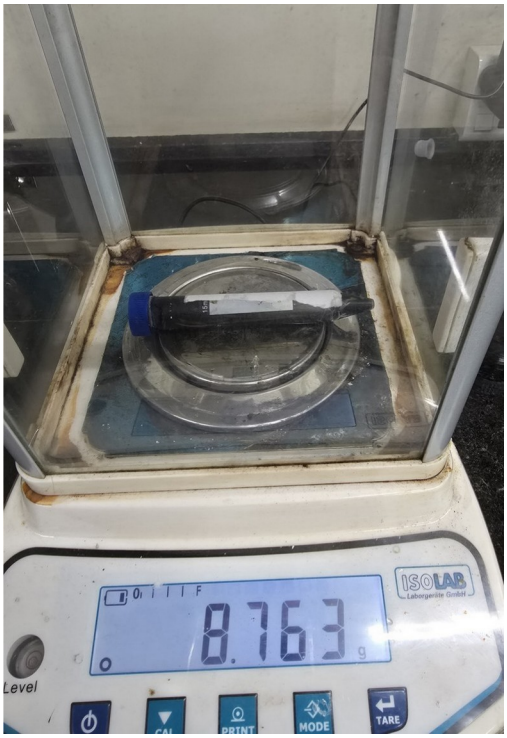


Figure 3.4: Weighing of the crude extract using an analytical balance.

3.6.9 Determination of Percentage Yield

The percentage yield of the crude extract was calculated on the basis of the weight of the dried leaf powder used for extraction:

$$\text{Yield (\%)} = (W_2 / W_1) \times 100 \qquad (3.1)$$

where W_1 is the weight of the dried leaf powder (g) and W_2 is the weight of the dried crude extract (g). In the present study, W_1 = 400 g and W_2 = 20.22 g. Therefore, yield (%) = (20.22 / 400) × 100 = **5.06% (w/w)**. The collection, drying and extraction data are summarised in Table 3.3.

Parameter	Observation / value
Place of collection	Birulia, Savar, Dhaka, Bangladesh
Date of collection	27 February 2026
Plant part used	Mature leaves, plucked directly from the tree
Weight of fresh leaves	Approximately 3 kg (3000 g)
Weight of shade-dried leaves	Approximately 900 g
Loss of weight (moisture) on drying	About 70% of the fresh weight
Weight of leaf powder extracted (W_1)	400 g
Extraction solvent and volume	98% ethanol, 800 mL (1:2, w/v)
Extraction method and duration	Cold maceration at room temperature, 3 days (72 h)
Concentration of the filtrate	Rotary vacuum evaporator (40–45 °C, 60 rpm)
Weight of dried crude extract (W_2)	20.22 g
Percentage yield (w/w, dry powder basis)	**5.06%**

Table 3.3: Summary of the collection, drying and extraction data of *P. guajava* leaves

3.7 Preparation of Stock Solutions and Working Concentrations

Stock solutions of the extract and of the reference standards were prepared by dissolving accurately weighed quantities in methanol and mixing on a vortex mixer until completely dissolved. Working solutions of lower concentration were prepared either by two-fold serial dilution (each solution being diluted with an equal volume of methanol to give half its concentration) or by direct dilution according to the relationship $C_1V_1 = C_2V_2$. Fresh solutions were prepared on the day of analysis. The concentrations used in each assay are summarised in Table 3.4, and the detailed procedures are described in Chapter 4.

Assay	Sample	Stock solution	Working concentrations (µg/mL)
DPPH	Extract	1 mg/mL in methanol	200, 100, 50, 25, 12.5
DPPH	Ascorbic acid	Prepared in methanol	200, 100, 50, 25, 12.5
TPC	Extract	1 mg in 5 mL methanol (200 µg/mL)	200, 100, 50, 25
TPC	Gallic acid	Prepared in methanol	200, 100, 50, 25
TFC	Extract	10 mg in 10 mL methanol (1 mg/mL)	100, 75, 50, 25
TFC	Quercetin	5 mg in 5 mL methanol (1 mg/mL)	100, 75, 50, 25

Table 3.4: Stock solutions and working concentrations used in each assay

3.8 Data Analysis

Absorbance readings were recorded directly from the spectrophotometer. For the TFC assay, which was performed in triplicate, results are expressed as mean ± standard deviation (SD) of the absorbance and as mean ± standard error of the mean (SEM) of the calculated content. The final TPC and TFC values are reported as the mean ± SEM of the values calculated at the different extract concentrations. Calibration curves were constructed by least-squares linear regression, and the coefficient of determination (R^2) was used to assess linearity. IC_{50} values were calculated from the regression equation of the dose–response curve by setting the percentage inhibition to 50%. The relationship between the phenolic or flavonoid content and the DPPH scavenging activity was assessed using Pearson's correlation coefficient (r). Calculations and regression analyses were performed using standard spreadsheet and statistical software.

CHAPTER 4

EVALUATION OF ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENT

4.1 Introduction

This chapter describes the principles, reagents, procedures and calculations of the three *in vitro* assays performed on the ethanolic leaf extract of *P. guajava*, namely the DPPH free radical scavenging assay, the Folin–Ciocalteu assay for total phenolic content and the aluminium chloride assay for total flavonoid content, and presents the results obtained in each assay.

4.2 DPPH Free Radical Scavenging Assay

4.2.1 Principle

DPPH is a stable free radical because its unpaired electron is delocalised over the whole molecule; for the same reason it does not dimerise. Its methanolic solution is deep violet and shows maximum absorbance at 517 nm. When DPPH$^{\bullet}$ is mixed with a substance that can donate a hydrogen atom or an electron (AH), it is converted to its reduced, non-radical form (DPPH–H), and the violet colour fades to pale yellow (Figure 4.1). The decrease in absorbance at 517 nm, measured with a UV–Visible spectrophotometer, is proportional to the radical-scavenging capacity of the sample *(Brand-Williams et al., 1995; Molyneux, 2004; Kedare & Singh, 2011)*. The activity is expressed as percentage inhibition and as the IC_{50} value, which is the concentration of the sample required to scavenge 50% of the DPPH radicals; a lower IC_{50} indicates stronger antioxidant activity.

$$DPPH^{\bullet}\ (violet) + AH \rightarrow DPPH\text{–}H\ (pale\ yellow) + A^{\bullet}$$

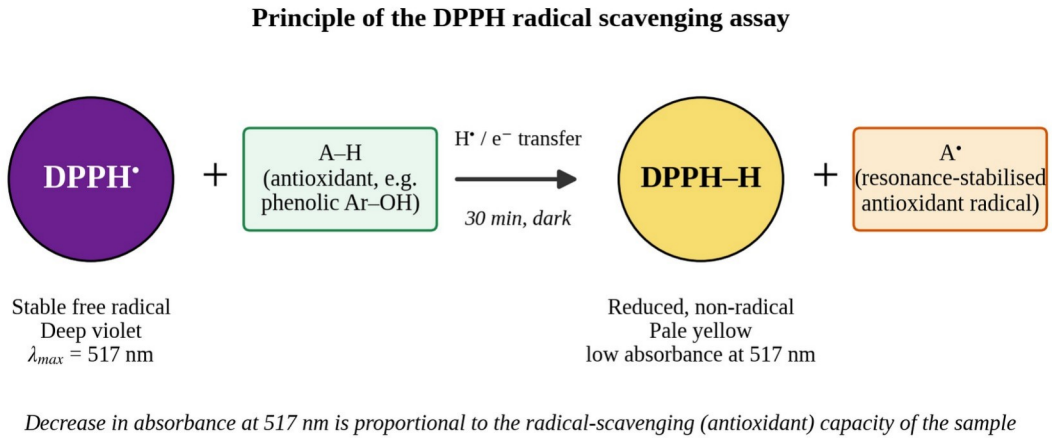


Figure 4.1: Principle of the DPPH radical scavenging assay.

4.2.2 Materials and Reagents

- DPPH (2,2-diphenyl-1-picrylhydrazyl)

- Ascorbic acid (standard antioxidant)
- Methanol
- Crude ethanolic extract of *P. guajava* leaves
- Test tubes and test tube rack
- Pipettes and micropipettes
- Amber-coloured volumetric flasks
- Aluminium foil / light-proof box (dark environment)
- UV–Visible spectrophotometer

4.2.3 Preparation of DPPH Solution (0.1 mM)

A 0.1 mM DPPH solution was prepared by dissolving 4 mg of DPPH (molecular weight 394.32 g/mol) in methanol and making up the volume to 100 mL in an amber-coloured volumetric flask (0.1 mmol/L × 0.1 L × 394.32 mg/mmol ≈ 3.94 mg ≈ 4 mg). The solution was freshly prepared and protected from light by keeping it in a light-proof box until use, because DPPH is light-sensitive.

4.2.4 Preparation of Standard Solutions (Ascorbic Acid)

Ascorbic acid was used as the standard antioxidant (positive control). A stock solution of ascorbic acid was prepared in methanol and diluted to give working solutions of 200, 100, 50, 25 and 12.5 µg/mL by two-fold serial dilution with methanol (Table 4.1).

4.2.5 Preparation of Extract Solutions

An accurately weighed quantity of the crude extract was dissolved in methanol to obtain a stock solution of 1 mg/mL (1000 µg/mL). From this stock, a 200 µg/mL solution was prepared using the relationship $C_1V_1 = C_2V_2$, and further concentrations of 100, 50, 25 and 12.5 µg/mL were obtained by two-fold serial dilution with methanol, as shown in Table 4.1.

Solution	Concentration (µg/mL)	Preparation
S1	200	Prepared from the 1000 µg/mL stock solution ($C_1V_1 = C_2V_2$)
S2	100	S1 diluted with an equal volume of methanol (1:1)
S3	50	S2 diluted with an equal volume of methanol (1:1)
S4	25	S3 diluted with an equal volume of methanol (1:1)
S5	12.5	S4 diluted with an equal volume of methanol (1:1)

Table 4.1: Serial dilution scheme used for the extract and ascorbic acid in the DPPH assay

4.2.6 Preparation of Control and Blank

Control: 2.4 mL of the 0.1 mM DPPH solution was mixed with 1.6 mL of methanol (no sample). **Blank:** methanol alone was used to set the zero absorbance of the spectrophotometer.

4.2.7 Assay Procedure

The DPPH radical scavenging activity was determined according to the method described by *Phuyal et al. (2020)*, which is based on that of *Brand-Williams et al. (1995)*. Briefly, 1.6 mL of each concentration of the extract or ascorbic acid was mixed with 2.4 mL of the freshly prepared 0.1 mM DPPH solution. The mixtures were shaken well and incubated in the dark at room temperature for 30 minutes. The absorbance of each mixture and of the control was then measured at 517 nm using a UV–Visible spectrophotometer against methanol as the blank (Figure 4.2).

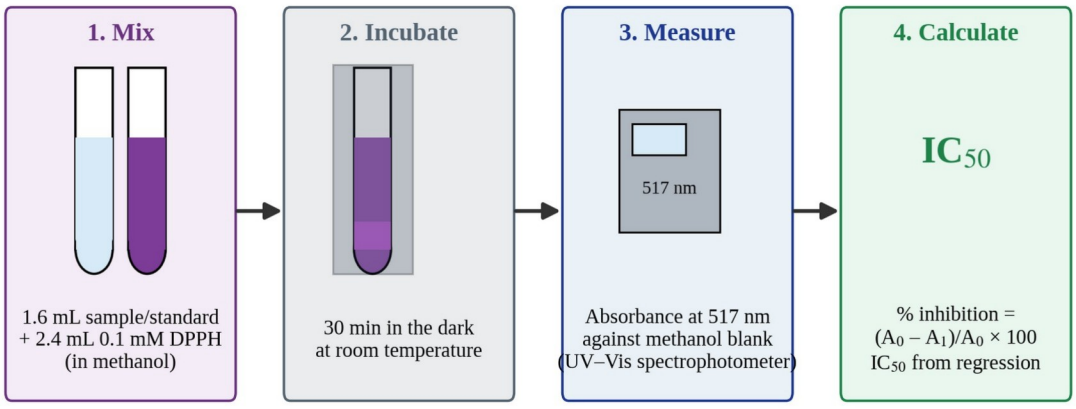


Figure 4.2: Schematic representation of the DPPH assay procedure.

4.2.8 Calculation of Percentage Inhibition and IC_{50}

The percentage of DPPH radical scavenging (inhibition) was calculated using the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0] \times 100 \quad (4.1)$$

where A_0 is the absorbance of the control (DPPH solution without sample) and A_1 is the absorbance in the presence of the extract or standard. The percentage inhibition was plotted against concentration, and the IC_{50} value was obtained from the regression equation of the linear portion of the dose–response curve by setting y = 50. For the extract, which showed a linear response over the whole concentration range (12.5–200 µg/mL), linear regression of % inhibition on concentration (y = mx + c) was used, so that IC_{50} = (50 − c)/m. For ascorbic acid, whose response increased logarithmically and approached a plateau above 100 µg/mL, linear regression of % inhibition on $\log_{10}$(concentration) over the linear range (12.5–100 µg/mL) was used, so that $IC_{50} = 10^{(50 - c)/m}$.

4.2.9 Results of the DPPH Assay

The absorbance of the DPPH control solution (A_0) was **0.570**. Both ascorbic acid and the extract reduced the absorbance of the DPPH solution in a concentration-dependent manner. The absorbance values and the calculated percentage inhibition for ascorbic acid are presented in Table 4.2 and Figure 4.3.

Concentration (µg/mL)	Absorbance (A_1)	% Inhibition
12.5	0.295	48.25
25	0.214	62.46
50	0.138	75.79
100	0.082	85.61
200	0.058	89.82

Table 4.2: Concentration versus percentage inhibition of DPPH by ascorbic acid (standard)

Note: A_0 (control) = 0.570. Sample calculation at 12.5 µg/mL: % inhibition = [(0.570 − 0.295)/0.570] × 100 = 48.25%.

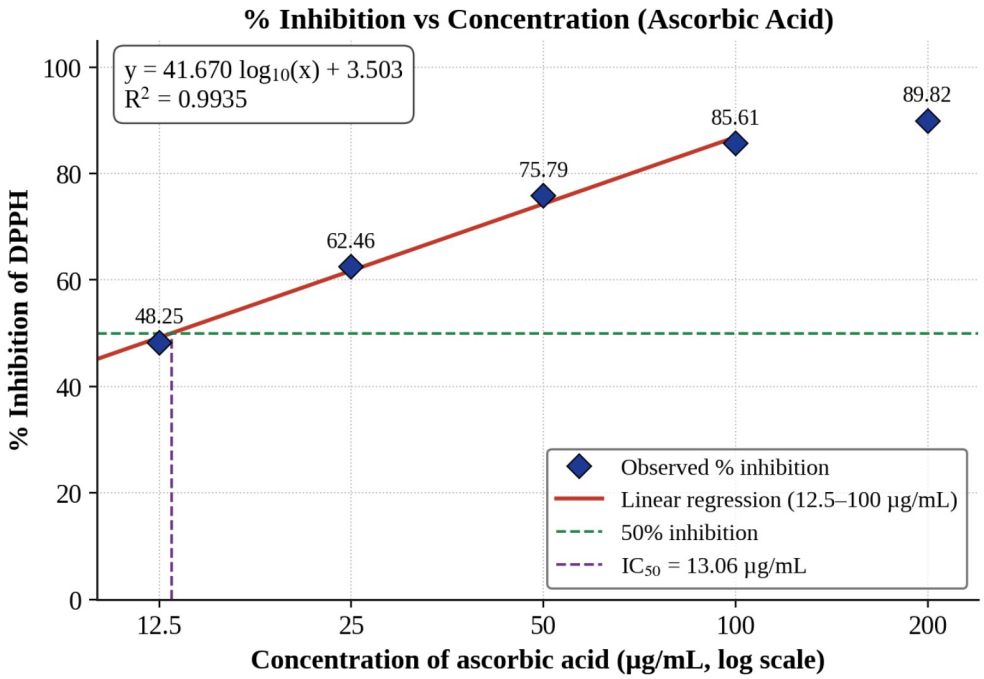


Figure 4.3: Concentration versus percentage inhibition curve used to determine the IC_{50} value of ascorbic acid.

Ascorbic acid showed strong radical-scavenging activity, with 48.25% inhibition even at the lowest concentration (12.5 µg/mL), rising to 89.82% at 200 µg/mL. The regression equation for the linear portion of the curve was $y = 41.670 \log_{10}(x) + 3.503$ ($R^2 = 0.9935$). Setting $y = 50$ gives $\log_{10}(IC_{50}) = (50 - 3.503)/41.670 = 1.1159$; therefore the **$IC_{50}$ of ascorbic acid = 13.06 µg/mL**. This value lies between the two lowest concentrations tested (12.5 and 25 µg/mL), in agreement with the observed data; simple linear interpolation between these two points gives a similar value (14.04 µg/mL).

The results for the ethanolic leaf extract of *P. guajava* are shown in Table 4.3 and Figure 4.4.

Concentration (µg/mL)	Absorbance (A_1)	% Inhibition
12.5	0.493	13.51
25	0.472	17.19
50	0.429	24.74
100	0.369	35.26
200	0.249	56.32

Table 4.3: Concentration versus percentage inhibition of DPPH by the ethanolic leaf extract of *P. guajava*

Note: A_0 (control) = 0.570. Sample calculation at 200 µg/mL: % inhibition = [(0.570 − 0.249)/0.570] × 100 = 56.32%.

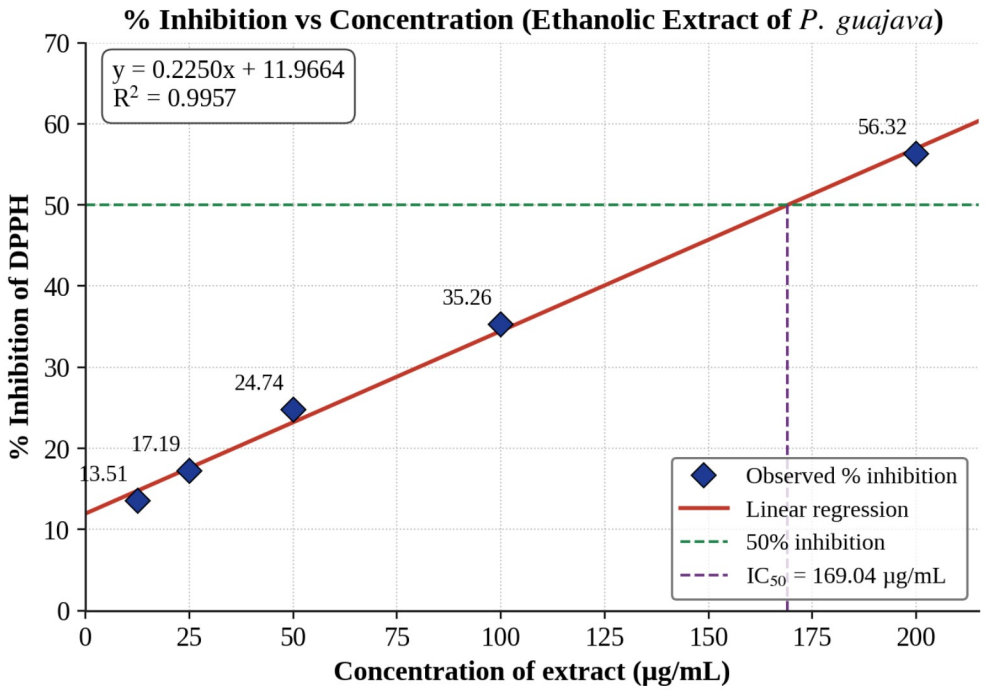


Figure 4.4: Concentration versus percentage inhibition curve used to determine the IC_{50} value of the ethanolic leaf extract of *P. guajava*.

The extract exhibited clear concentration-dependent DPPH scavenging activity, with percentage inhibition increasing from 13.51% at 12.5 µg/mL to 56.32% at 200 µg/mL. The dose–response relationship was linear over the whole concentration range, with the regression equation $y = 0.2250x + 11.9664$ ($R^2 = 0.9957$). Setting $y = 50$ gives $x = (50 - 11.9664)/0.2250$; therefore the **$IC_{50}$ of the extract = 169.04 µg/mL**. The IC_{50} values are summarised in Table 4.4 and compared in Figure 4.5.

Sample	Regression equation	R^2	IC_{50} (µg/mL)
Ascorbic acid (standard)	$y = 41.670 \log_{10}(x) + 3.503$	0.9935	13.06
P. guajava leaf extract	$y = 0.2250x + 11.9664$	0.9957	169.04

Table 4.4: IC_{50} values of ascorbic acid and the ethanolic leaf extract of *P. guajava* in the DPPH assay

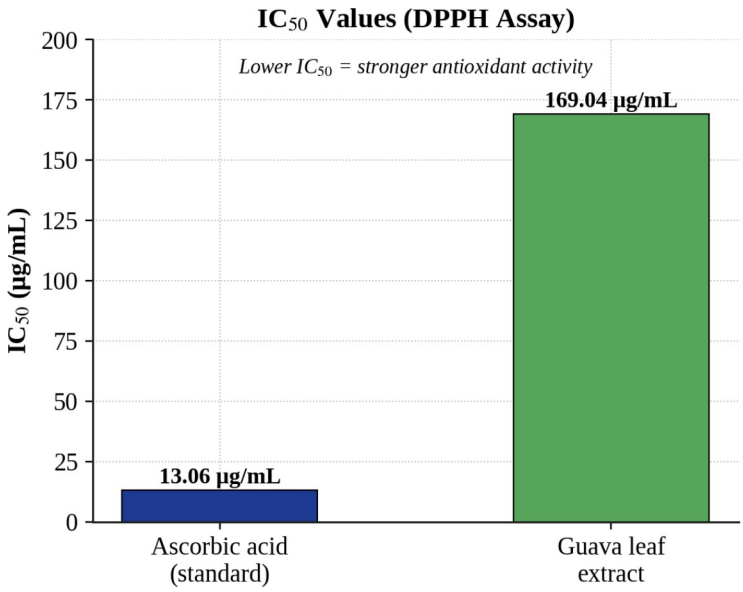


Figure 4.5: Comparison of the IC_{50} values of ascorbic acid and the ethanolic leaf extract of *P. guajava* (lower IC_{50} indicates stronger activity).

At every concentration tested, the percentage inhibition produced by the extract was lower than that of ascorbic acid, and the IC_{50} of the extract was approximately 12.9 times higher than that of the standard (169.04 versus 13.06 µg/mL). Nevertheless, the extract achieved more than 50% inhibition of DPPH radicals at 200 µg/mL and showed a consistent, linear increase in activity with concentration, indicating that it contains constituents capable of donating hydrogen atoms or electrons to free radicals.

4.3 Determination of Total Phenolic Content (TPC)

4.3.1 Principle

The total phenolic content was determined by the Folin–Ciocalteu method *(Singleton & Rossi, 1965; Singleton et al., 1999)*. Phenolic compounds possess one or more hydroxyl groups attached to an aromatic ring. In the presence of sodium carbonate, the medium becomes alkaline (pH about 10) and the phenolic hydroxyl groups dissociate to form phenolate anions, which are strong reducing agents. The phenolate anions reduce the yellow phosphomolybdate–phosphotungstate complex of the Folin–Ciocalteu reagent, and the molybdenum(VI) is reduced to molybdenum(V), producing a blue-coloured complex (Figure 4.6). The intensity of the blue colour, measured at 765 nm, is proportional to the amount of phenolic compounds present, and the result is expressed as gallic acid equivalents *(Ainsworth & Gillespie, 2007)*.

$$\text{Phenols} + Na_2CO_3 \rightarrow \text{Phenolate ions (complete ionisation)}$$

Phenolate ions + FC reagent (Mo^{6+}, yellow) → Molybdenum blue (Mo^{5+}, blue)

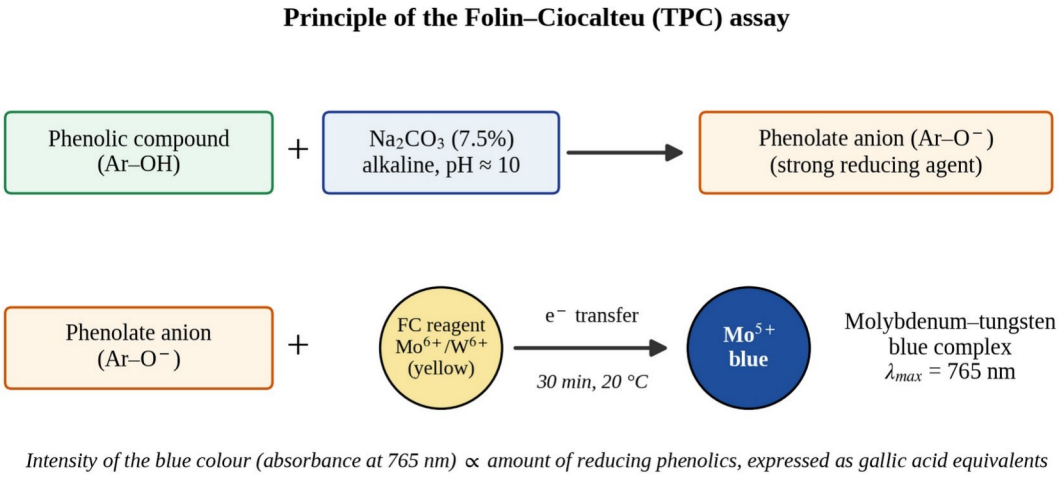


Figure 4.6: Principle of the Folin–Ciocalteu assay for total phenolic content.

The method described by *Phuyal et al. (2020)* and *Hatami et al. (2014)*, as adapted in the laboratory manual, was followed in this study.

4.3.2 Materials and Reagents

- Crude ethanolic extract of *P. guajava* leaves
- Folin–Ciocalteu reagent (diluted 10-fold)
- Sodium carbonate solution (7.5% w/v)
- Gallic acid (standard for the calibration curve)
- Methanol and distilled water
- UV–Visible spectrophotometer
- Pipettes, micropipettes, test tubes, test tube rack and aluminium foil

4.3.3 Preparation of Folin–Ciocalteu Reagent (10-fold Diluted)

The commercial Folin–Ciocalteu reagent was diluted 10-fold by mixing 10 mL of the reagent with distilled water and making up the volume to 100 mL (1:10, v/v). The diluted reagent was prepared fresh and protected from light.

4.3.4 Preparation of Sodium Carbonate Solution (7.5% w/v)

Sodium carbonate (7.5 g) was dissolved in distilled water and the volume was made up to 100 mL to obtain a 7.5% (w/v) solution.

4.3.5 Preparation of Gallic Acid Standard Solutions

A stock solution of gallic acid was prepared in methanol, from which standard solutions of 200, 100, 50 and 25 µg/mL were prepared by two-fold serial dilution with methanol.

4.3.6 Preparation of Extract Solutions

For the preparation of the stock sample, 1 mg of the extract was dissolved in 5 mL of methanol to obtain a concentration of 200 µg/mL. This solution was serially diluted (1:1) with methanol to obtain extract concentrations of 100, 50 and 25 µg/mL.

4.3.7 Assay Procedure

1. 1.0 mL of each extract solution (25–200 µg/mL) and of each gallic acid standard solution (25–200 µg/mL) was taken in separate test tubes.
2. 5 mL of the 10-fold diluted Folin–Ciocalteu reagent was added to each test tube.
3. 5 mL of 7.5% sodium carbonate solution was then added, and the contents were mixed thoroughly.
4. The test tubes were incubated for 30 minutes at 20 °C to allow the reaction to complete.
5. The absorbance was measured at 765 nm using a UV–Visible spectrophotometer against the blank.
6. The blank solution contained methanol in place of the sample.
7. A calibration curve was constructed by plotting the absorbance (y-axis) against the concentration of gallic acid (x-axis).

4.3.8 Calculation of Total Phenolic Content

The concentration of gallic acid equivalents in each extract solution was obtained from the calibration curve ($x = (y - c)/m$), and the total phenolic content was calculated using the following formula:

$$C = (c \times V) / m \tag{4.2}$$

where C = total phenolic content (mg GAE/g of dry extract); c = concentration of gallic acid obtained from the calibration curve (mg/mL); V = volume of the sample solution used (here, 1 mL); and m = weight of the extract contained in that volume (g), for example 200 µg = 0.0002 g for the 200 µg/mL solution.

4.3.9 Results of the TPC Assay

The absorbance values of the gallic acid standard solutions are presented in Table 4.5, and the resulting calibration curve is shown in Figure 4.7.

Concentration of gallic acid (µg/mL)	Absorbance at 765 nm
25	0.498
50	0.662
100	0.997
200	1.897

Table 4.5: Absorbance of gallic acid standard solutions at 765 nm

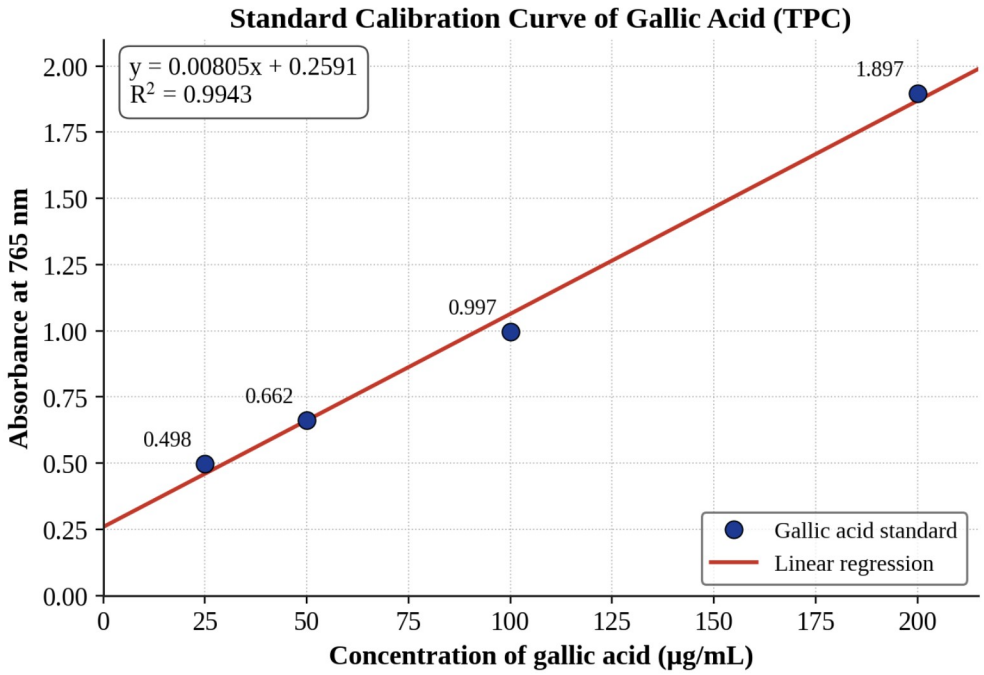


Figure 4.7: Standard calibration curve of gallic acid for the determination of total phenolic content.

The calibration curve was linear over the range 25–200 µg/mL, with the regression equation **y = 0.00805x + 0.2591** and a coefficient of determination **R^2 = 0.9943**, indicating good linearity. The absorbance values of the extract solutions are given in Table 4.6 and Figure 4.8.

Concentration of extract (µg/mL)	Absorbance at 765 nm
25	0.359
50	0.488
100	0.653
200	0.790

Table 4.6: Absorbance of the ethanolic leaf extract of *P. guajava* at 765 nm (TPC assay)

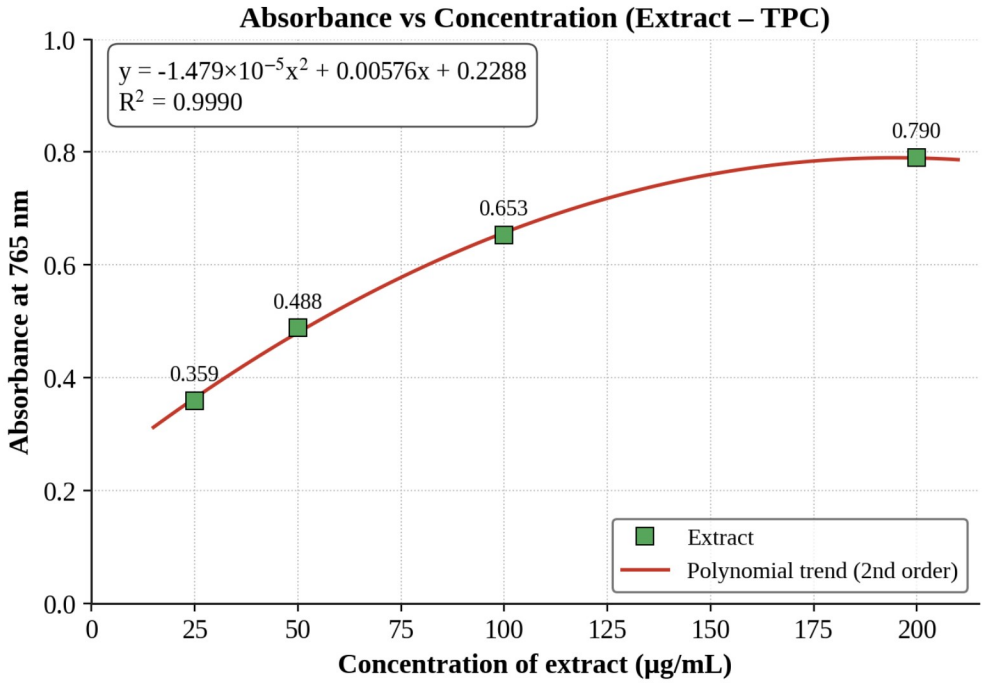


Figure 4.8: Absorbance of the extract at different concentrations in the total phenolic content assay.

The absorbance of the extract increased with concentration, although the increase became smaller at higher concentrations (second-order polynomial trend: $y = -1.479 \times 10^{-5}x^2 + 0.00576x + 0.2288$; $R^2 = 0.9990$). The gallic acid equivalent concentration and the total phenolic content calculated at each concentration are presented in Table 4.7.

Extract conc. (µg/mL)	Weight of extract in 1 mL, m (g)	Absorbance (y)	GAE from curve, x (µg/mL)	c (mg/mL)	TPC (mg GAE/g extract)
25	2.5×10^{-5}	0.359	12.41	0.01241	496.46
50	5.0×10^{-5}	0.488	28.44	0.02844	568.86
100	1.0×10^{-4}	0.653	48.95	0.04895	489.49
200	2.0×10^{-4}	0.790	65.97	0.06597	329.87
Mean ± SEM (n = 4)					**471.17 ± 50.40**

Table 4.7: Calculation of the total phenolic content of the ethanolic leaf extract of *P. guajava*

Note: GAE = gallic acid equivalent; x = (y − 0.2591)/0.00805; c = x/1000; V = 1 mL; TPC = c × V/m. SD = 100.80 mg GAE/g.

Sample calculation (200 µg/mL extract solution):

Absorbance (y) = 0.790
x = (0.790 − 0.2591) / 0.00805 = 65.97 µg/mL = 0.06597 mg/mL
C = (c × V)/m = (0.06597 mg/mL × 1 mL) / 0.0002 g = 329.87 mg GAE/g extract

The calculated TPC values ranged from 329.87 to 568.86 mg GAE/g at the four extract concentrations. The average total phenolic content of the ethanolic leaf extract of *P. guajava* was found to be **471.17 ± 50.40 mg GAE/g of dry extract** (mean ± SEM), indicating that the extract is rich in phenolic compounds.

4.4 Determination of Total Flavonoid Content (TFC)

4.4.1 Principle

The total flavonoid content was determined by the aluminium chloride colorimetric method *(Chang et al., 2002)*. Aluminium chloride forms stable acid complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols, and acid-labile complexes with ortho-dihydroxyl groups in ring A or B of flavonoids. Potassium acetate is added to stabilise the complex. The formation of the Al^{3+}–flavonoid complex produces a yellow colour whose absorbance, measured at 415 nm, is proportional to the flavonoid content (Figure 4.9). Quercetin was used as the standard, and the results were expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g) *(Pękal & Pyrzynska, 2014)*.

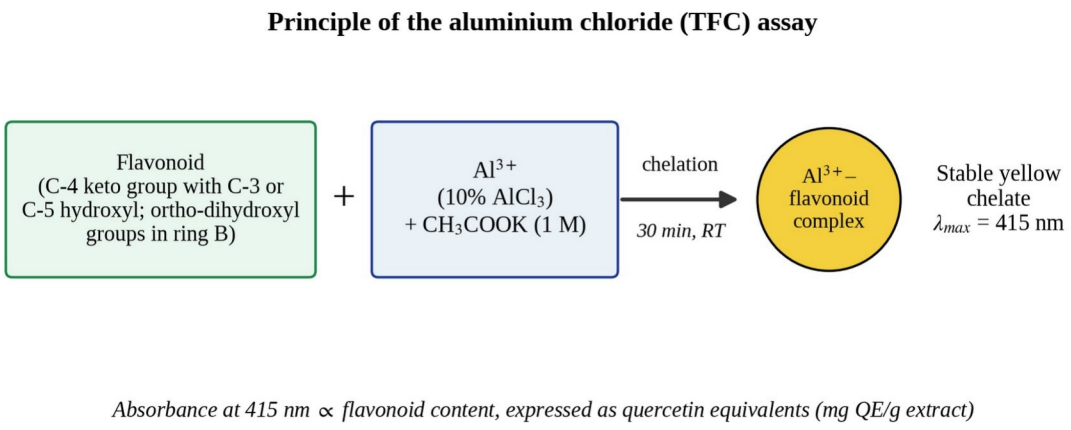


Figure 4.9: Principle of the aluminium chloride assay for total flavonoid content.

4.4.2 Materials and Reagents

- Crude ethanolic extract of *P. guajava* leaves
- Methanol
- Aluminium chloride ($AlCl_3$), 10% w/v
- Potassium acetate (CH_3COOK), 1 M
- Quercetin standard solution (for the calibration curve)
- UV–Visible spectrophotometer and cuvettes
- Distilled water
- Pipettes, micropipettes and test tubes

4.4.3 Preparation of Aluminium Chloride Solution (10% w/v)

A 10% (w/v) aluminium chloride solution was prepared by dissolving 250 mg of aluminium chloride hexahydrate in distilled water and making up the volume to 2.5 mL.

4.4.4 Preparation of Potassium Acetate Solution (1 M)

Potassium acetate has a molecular weight of 98.14 g/mol; therefore, a 1 M solution contains 9.8 g in 100 mL. For the present study, 0.49 g (490 mg) of potassium acetate was dissolved in 5 mL of distilled water to obtain a 1 M solution (0.49 g ÷ 98.14 g/mol = 0.005 mol in 0.005 L = 1 M).

4.4.5 Preparation of Quercetin Standard Solutions

A quercetin stock solution was prepared by dissolving 5 mg of quercetin in 5 mL of methanol to obtain a concentration of 1 mg/mL (1000 µg/mL). Standard solutions (1 mL each) of 25, 50, 75 and 100 µg/mL were prepared from the stock solution using the relationship $C_1V_1 = C_2V_2$, where C_1 = 1000 µg/mL, C_2 = the desired concentration and V_2 = 1 mL. For example, for 25 µg/mL: $V_1 = (25 \times 1)/1000 = 0.025$ mL = 25 µL. The volumes used are shown in Table 4.8.

Concentration (µg/mL)	Volume of stock solution added	Volume of methanol added
25	25 µL	975 µL
50	50 µL	950 µL
75	75 µL	925 µL
100	100 µL	900 µL

Table 4.8: Preparation of quercetin standard solutions (final volume 1 mL)

4.4.6 Preparation of Extract Solutions

A stock solution of the extract was prepared by dissolving 10 mg of the extract in 10 mL of methanol to obtain a concentration of 1 mg/mL (1000 µg/mL). Extract solutions of 25, 50, 75 and 100 µg/mL were then prepared from this stock in the same way as the quercetin standards (Table 4.8).

4.4.7 Assay Procedure

1. 0.5 mL of each extract or quercetin standard solution was mixed with 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 4.3 mL of distilled water to give a final volume of 5 mL.
2. The mixture was incubated for 30 minutes at room temperature.
3. The absorbance of the reaction mixture was measured at 415 nm against the blank (methanol) using a UV–Visible spectrophotometer.
4. Each sample and standard was analysed in triplicate, and the mean absorbance was used for calculation.
5. A calibration curve was constructed by plotting the quercetin concentrations (x-axis) against their corresponding absorbance values (y-axis).

4.4.8 Calculation of Total Flavonoid Content

The quercetin-equivalent concentration of each extract solution was obtained from the calibration curve (the value obtained from the curve is in µg/mL and was converted to mg/mL), and the total flavonoid content was calculated using the formula:

$$C = c \times (V / m) \tag{4.3}$$

where C = total flavonoid content (mg QE/g of dry extract); c = concentration of quercetin obtained from the calibration curve (mg/mL); V = volume of the extract solution used in the assay (here, 0.5 mL); and m = weight of the extract contained in that volume (g).

4.4.9 Results of the TFC Assay

The triplicate absorbance values of the quercetin standard solutions are presented in Table 4.9, and the corresponding calibration curve is shown in Figure 4.10.

Concentration (µg/mL)	Abs 1	Abs 2	Abs 3	Mean absorbance ± SD
25	0.142	0.140	0.129	0.1370 ± 0.0070
50	0.214	0.186	0.214	0.2047 ± 0.0162
75	0.296	0.316	0.310	0.3073 ± 0.0103
100	0.382	0.395	0.388	0.3883 ± 0.0065

Table 4.9: Absorbance of quercetin standard solutions at 415 nm (n = 3)

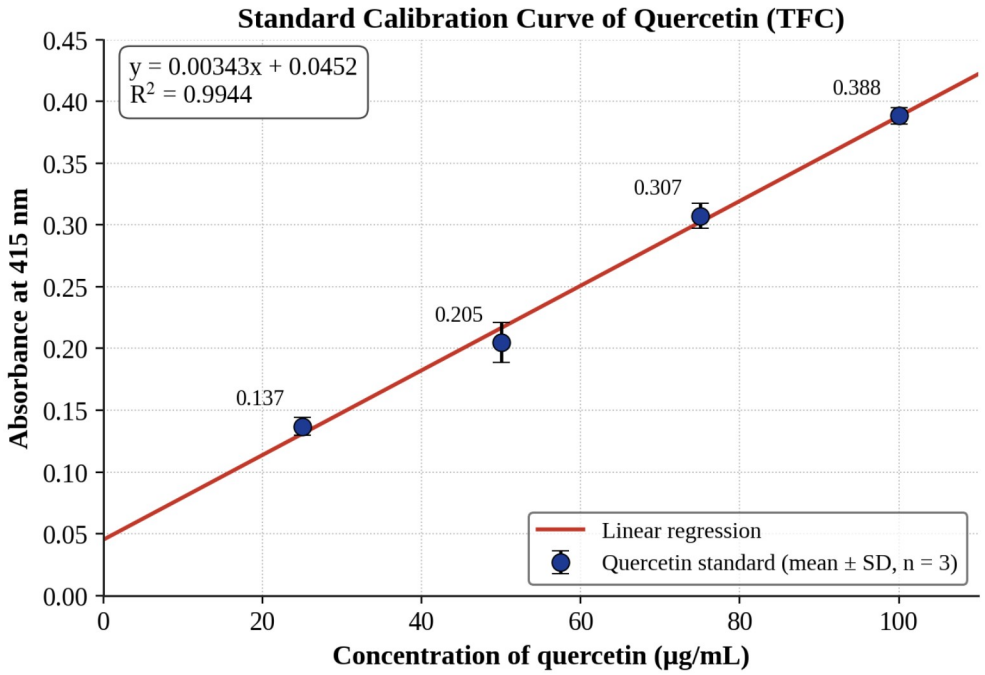


Figure 4.10: Standard calibration curve of quercetin for the determination of total flavonoid content.

The quercetin calibration curve was linear over the range 25–100 µg/mL, with the regression equation **y = 0.00343x + 0.0452** and **R^2 = 0.9944**. The triplicate absorbance values of the extract are given in Table 4.10 and Figure 4.11.

Concentration (µg/mL)	Abs 1	Abs 2	Abs 3	Mean absorbance ± SD
25	0.053	0.055	0.053	0.0537 ± 0.0012
50	0.069	0.066	0.071	0.0687 ± 0.0025
75	0.081	0.083	0.082	0.0820 ± 0.0010
100	0.096	0.097	0.095	0.0960 ± 0.0010

Table 4.10: Absorbance of the ethanolic leaf extract of *P. guajava* at 415 nm (TFC assay, n = 3)

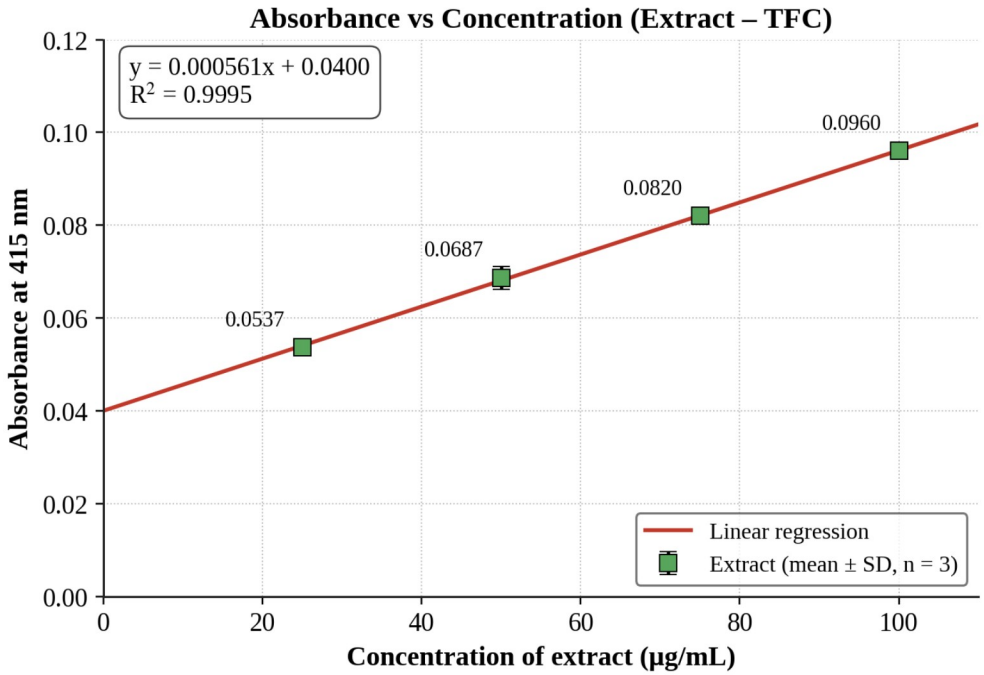


Figure 4.11: Absorbance of the extract at different concentrations in the total flavonoid content assay.

The absorbance of the extract increased linearly with concentration (y = 0.000561x + 0.0400; R^2 = 0.9995), and the triplicate readings showed good repeatability. The calculated total flavonoid content at each concentration is shown in Table 4.11.

Extract conc. (µg/mL)	Mean absorbance (y)	QE from curve, x (µg/mL)	c (mg/mL)	Weight of extract in 0.5 mL, m (g)	TFC (mg QE/g) mean ± SEM
25	0.0537	2.48	0.00248	1.25×10^{-5}	99.22 ± 7.78
50	0.0687	6.86	0.00686	2.50×10^{-5}	137.16 ± 8.48
75	0.0820	10.75	0.01075	3.75×10^{-5}	143.32 ± 2.25
100	0.0960	14.83	0.01483	5.00×10^{-5}	148.35 ± 1.68
Mean ± SEM (n = 4)					**132.01 ± 11.17**

Table 4.11: Calculation of the total flavonoid content of the ethanolic leaf extract of *P. guajava*

Note: QE = quercetin equivalent; x = (y − 0.0452)/0.00343; c = x/1000; V = 0.5 mL; TFC = c × V/m; values at each concentration are mean ± SEM of the three replicate determinations. SD of the four concentration means = 22.33 mg QE/g.

Sample calculation (100 µg/mL extract solution):

Mean absorbance (y) = 0.0960
x = (0.0960 − 0.0452) / 0.00343 = 14.83 µg/mL = 0.01483 mg/mL
m = 100 µg/mL × 0.5 mL = 50 µg = 5.0×10^{-5} g

$C = c \times (V/m) = 0.01483\ mg/mL \times (0.5\ mL / 5.0 \times 10^{-5}\ g) = 148.35\ mg\ QE/g\ extract$

The average total flavonoid content of the ethanolic leaf extract of *P. guajava* was found to be **132.01 ± 11.17 mg QE/g of dry extract** (mean ± SEM). The flavonoid content therefore corresponds to about 28% of the total phenolic content, suggesting that, in addition to flavonoids, other phenolic compounds such as tannins and phenolic acids make a substantial contribution to the phenolic pool of guava leaves.

4.5 Relationship Between Phenolic/Flavonoid Content and DPPH Scavenging Activity

To examine whether the radical-scavenging activity of the extract was associated with its phenolic and flavonoid constituents, the percentage DPPH inhibition at each extract concentration was compared with the phenolic (µg GAE/mL) and flavonoid (µg QE/mL) contents measured in extract solutions of the same concentration (Table 4.12).

Extract conc. (µg/mL)	DPPH inhibition (%)	Phenolics (µg GAE/mL)	Flavonoids (µg QE/mL)
25	17.19	12.41	2.48
50	24.74	28.44	6.86
75	–	–	10.75
100	35.26	48.95	14.83
200	56.32	65.97	–

Table 4.12: DPPH inhibition and phenolic/flavonoid content of the extract at matched concentrations

Note: – = concentration not tested in that assay.

A strong positive linear relationship was observed between DPPH inhibition and phenolic content (Pearson $r = 0.971$, $p = 0.029$, $n = 4$) and between DPPH inhibition and flavonoid content ($r = 0.997$, $p = 0.046$, $n = 3$). These results indicate that the radical-scavenging activity of the extract increases in parallel with its phenolic and flavonoid content. Because both variables increase with extract concentration, this relationship reflects the dose-dependence of activity within a single extract rather than a comparison among different extracts; nevertheless, it supports the view that phenolic compounds and flavonoids contribute to the antioxidant activity observed.

4.6 Summary of Results

Parameter	Ethanolic leaf extract	Standard
DPPH scavenging, IC_{50} (µg/mL)	169.04	Ascorbic acid: 13.06
Maximum DPPH inhibition (at 200 µg/mL)	56.32%	Ascorbic acid: 89.82%
Total phenolic content (mg GAE/g extract)	471.17 ± 50.40	Gallic acid (R^2 = 0.9943)
Total flavonoid content (mg QE/g extract)	132.01 ± 11.17	Quercetin (R^2 = 0.9944)
TFC/TPC ratio	28.0%	–

Table 4.13: Summary of the antioxidant activity and phenolic/flavonoid contents of the ethanolic leaf extract of *P. guajava*

Note: TPC and TFC values are mean ± SEM of the values calculated at the different extract concentrations.

CHAPTER 5
DISCUSSION

The present study evaluated the *in vitro* antioxidant potential of the ethanolic leaf extract of *Psidium guajava* L. using the DPPH radical scavenging assay and quantified its total phenolic and total flavonoid contents. Because oxidative stress caused by excess reactive oxygen species is associated with the development of cancer, diabetes, cardiovascular and neurodegenerative diseases and ageing *(Valko et al., 2007; Pham-Huy et al., 2008)*, plant materials capable of scavenging free radicals are of considerable pharmaceutical interest. The main findings are discussed below.

5.1 Interpretation of the DPPH Results

The DPPH assay showed that the ethanolic leaf extract of *P. guajava* scavenged DPPH radicals in a clear concentration-dependent manner, with inhibition increasing from 13.51% at 12.5 µg/mL to 56.32% at 200 µg/mL. The strong linear dose–response relationship (R^2 = 0.9957) indicates that the scavenging effect is directly attributable to constituents of the extract. The IC_{50} of the extract (169.04 µg/mL) was about 13 times higher than that of the reference antioxidant ascorbic acid (13.06 µg/mL), meaning that the extract was less potent than the pure standard.

This difference is expected, because ascorbic acid is a pure, highly efficient reducing agent that reacts rapidly with DPPH, whereas a crude extract is a complex mixture in which the active phenolic compounds are present together with many constituents that have little or no radical-scavenging activity, such as chlorophylls, waxes, lipids, sugars and structural materials *(Kedare & Singh, 2011)*. *Qian and Nihorimbere (2004)* and *Simamora et al. (2018)* likewise found ascorbic acid to be considerably more potent than guava leaf extracts. It should also be noted that the IC_{50} value obtained for ascorbic acid in this study (13.06 µg/mL) is of the same order as values reported by other workers, such as 21.36 µg/mL *(Simamora et al., 2018)*, which supports the validity of the assay conditions.

Several factors may explain why the activity of the present extract was lower than that reported for some other guava leaf extracts. First, near-absolute (98%) ethanol was used as the extraction solvent, whereas hydroethanolic solvents containing about 50% water extract more of the highly polar phenolics, such as tannins and flavonoid glycosides, and yield extracts with higher antioxidant activity *(Seo et al., 2014; Dang Thi et al., 2024)*. Secondly, ethanol efficiently co-extracts chlorophyll and other lipophilic pigments, as reflected by the dark green colour of the extract (Figure 3.3), which dilutes the active

constituents. Thirdly, large polymeric tannins react slowly with DPPH and may be sterically hindered from reaching the radical site, so that a 30-minute reaction time may underestimate their contribution *(Brand-Williams et al., 1995; Kedare & Singh, 2011)*. Finally, differences in variety, leaf maturity, season and growing conditions are known to influence the antioxidant activity of guava leaves *(Camarena-Tello et al., 2018; Sowmya & Usha Anandhi, 2020; Kumar et al., 2021)*.

5.2 Interpretation of the TPC Results

The Folin–Ciocalteu assay showed that the ethanolic extract contained a high level of phenolic compounds, with an average total phenolic content of 471.17 ± 50.40 mg GAE/g of dry extract. The gallic acid calibration curve showed good linearity (R^2 = 0.9943), supporting the reliability of the quantification. This value means that nearly half of the dry weight of the extract behaves as gallic acid-equivalent reducing phenolics, which is consistent with the well-documented richness of guava leaves in tannins, flavonoids and phenolic acids *(Gutiérrez et al., 2008; Díaz-de-Cerio et al., 2017; Kumar et al., 2021)*.

The TPC values calculated at the individual extract concentrations varied (329.87–568.86 mg GAE/g). The lowest value was obtained at the highest concentration (200 µg/mL), where the absorbance of the extract began to level off (Figure 4.8), while the absorbances at 25 and 50 µg/mL were slightly below that of the lowest gallic acid standard, so that these values were estimated by slight extrapolation of the calibration line. Reporting the mean of all concentrations, together with its standard error, therefore gives a more representative estimate than any single concentration. It should also be remembered that the Folin–Ciocalteu reagent measures total reducing capacity and can react with non-phenolic reducing substances such as ascorbic acid, reducing sugars and certain proteins *(Everette et al., 2010; Sánchez-Rangel et al., 2013)*. Guava leaves contain appreciable amounts of ascorbic acid *(Kumar et al., 2021)*, so the TPC value may be somewhat overestimated.

5.3 Interpretation of the TFC Results

The aluminium chloride assay revealed a total flavonoid content of 132.01 ± 11.17 mg QE/g of dry extract. The quercetin calibration curve was linear (R^2 = 0.9944), and the triplicate absorbance readings of the extract showed low variability. Flavonoids thus accounted for about 28% of the total phenolic content, indicating that non-flavonoid phenolics, particularly tannins and phenolic acids, constitute the larger part of the

phenolic fraction. This agrees with phytochemical studies showing that guava leaves contain large amounts of ellagitannins and gallic and ellagic acid derivatives in addition to flavonol glycosides such as guaijaverin, avicularin, isoquercitrin and hyperoside *(Díaz-de-Cerio et al., 2017; Kumar et al., 2021)*.

Because quercetin glycosides are the dominant flavonoids of guava leaves, quercetin is an appropriate standard for this plant. However, the aluminium chloride method mainly detects flavones and flavonols and responds only weakly to flavan-3-ols such as catechin and epicatechin, which are also present in guava leaves; therefore, the true flavonoid content may be underestimated *(Pękal & Pyrzynska, 2014; Shraim et al., 2021)*. On the other hand, the extract absorbances (0.054–0.096) were lower than that of the lowest quercetin standard, and pigments such as chlorophyll also absorb near 415 nm; without a separate sample blank, this background absorbance could lead to some overestimation *(Shraim et al., 2021)*. The lower TFC value calculated at 25 µg/mL, where the absorbance was closest to the intercept of the calibration line, reflects the greater relative uncertainty at very low absorbance.

5.4 Comparison with Previous Studies

The results of the present study are compared with selected published data on guava leaf extracts in Table 5.1. Direct comparison is limited because of differences in plant variety, extraction solvent and method, assay conditions and units (per gram of extract versus per gram of dry leaf); nevertheless, the comparison places the present findings in context.

Study	Extract / method	TPC	TFC	DPPH IC_{50} (µg/mL)
Qian & Nihorimbere (2004)	50% ethanol and water extracts	575.3 and 511.6 mg GAE/g	–	Less active than ascorbic acid
You et al. (2011)	Acetone leaf extract (most active of four solvents)	141.28 mg GAE/g	–	34.01
Fernandes et al. (2014)	Spray-dried hydroalcoholic extracts	–	–	7.96–8.11
Simamora et al. (2018)	Aqueous leaf extract	–	–	74.77 (ascorbic acid 21.36)
Hartati et al. (2020)	Ethanol (reflux) leaf extract	495.5 mg GAE/g	Up to 96.8 mg QE/g[a]	Strong activity (AAI)
Sowmya & *Usha Anandhi (2020)*	Ethanol (Soxhlet), two cultivars	Reported	Reported	229.56 and 270.24
Dang Thi et al. (2024)	Ultrasound-assisted, 51.9% ethanol	152.95 mg GAE/g DW	113.87 mg QE/g DW	Reported as mg TE/g
Present study	**Cold maceration, 98% ethanol**	**471.17 ± 50.40 mg GAE/g**	**132.01 ± 11.17 mg QE/g**	**169.04 (ascorbic acid 13.06)**

Table 5.1: Comparison of the present results with previously reported values for guava leaf extracts

Note: [a]Highest value, obtained for the n-hexane leaf extract. DW = dry weight of leaves; AAI = antioxidant activity index; TE = Trolox equivalent; – = not reported.

The TPC of the present extract (471.17 mg GAE/g) is comparable to the values reported for ethanolic and aqueous guava leaf extracts by *Qian and Nihorimbere (2004)* and *Hartati et al. (2020)*, and higher than that reported for acetone extracts by *You et al. (2011)*, confirming that guava leaves are an exceptionally rich source of phenolic compounds. The TFC (132.01 mg QE/g) is of the same order as the value obtained by *Dang Thi et al. (2024)* under optimised ultrasound-assisted conditions. The DPPH IC_{50} (169.04 µg/mL) lies within the wide range reported in the literature: it indicates lower activity than spray-dried hydroalcoholic, acetone and aqueous extracts *(Fernandes et al., 2014; You et al., 2011; Simamora et al., 2018)*, but higher activity than the Soxhlet ethanolic extracts of two Indian guava cultivars, for which IC_{50} values of 229.56 and 270.24 µg/mL were reported *(Sowmya & Usha Anandhi, 2020)*.

5.5 Relationship Between Phenolic Content and Antioxidant Activity

At matched concentrations, the DPPH inhibition produced by the extract increased linearly with its phenolic content (r = 0.971) and flavonoid content (r = 0.997), which suggests that these constituents are major contributors to the radical-scavenging activity. This agrees with *Seo et al. (2014)*, who found that the antioxidant capacity of guava leaf

extracts was strongly related to their phenolic content, and with *Hartati et al. (2020)*, who reported a significant positive correlation between the TPC of guava leaf extracts and their DPPH activity. However, the present extract combined a high TPC with only moderate DPPH activity. This apparent discrepancy can be explained by the fact that the Folin–Ciocalteu assay measures all reducing substances, whereas DPPH scavenging depends on the type and accessibility of the phenolics and on reaction kinetics; bulky tannins, for example, contribute strongly to TPC but react slowly with the sterically hindered DPPH radical *(Kedare & Singh, 2011; Hatami et al., 2014)*. In addition, because the correlation was calculated within a single extract, it mainly reflects the dose-dependence of both measurements; comparisons among several extracts or fractions would be required to establish a true structure–activity correlation.

5.6 Possible Mechanisms of Antioxidant Action

The antioxidant activity of guava leaf extract can be attributed mainly to its polyphenols, which act through several complementary mechanisms *(Rice-Evans et al., 1996; Leopoldini et al., 2011)*:

- **Hydrogen atom and electron donation:** phenolic hydroxyl groups of flavonoids, phenolic acids and tannins donate hydrogen atoms or electrons to free radicals such as DPPH$^{\bullet}$, forming resonance-stabilised phenoxyl radicals (HAT, SET-PT and SPLET mechanisms).
- **Favourable structural features of quercetin glycosides:** the catechol group in ring B and the 2,3-double bond conjugated with the 4-oxo group of quercetin and its glycosides stabilise the resulting radical and enhance scavenging ability *(Heim et al., 2002)*.
- **Multiple hydroxyl groups of tannins and gallic acid derivatives:** galloyl and hexahydroxydiphenoyl groups provide many donor sites and can also chelate pro-oxidant metal ions *(Dai & Mumper, 2010; Leopoldini et al., 2011)*.
- **Synergistic effects:** the combined action of different phenolics, flavonoids, triterpenoids and ascorbic acid present in the extract may produce greater overall activity than any single constituent *(Dai & Mumper, 2010; Kumar et al., 2021)*.

5.7 Therapeutic Relevance

Oxidative stress is a common pathological mechanism in many chronic diseases, including cancer, diabetes and its complications, cardiovascular disease and inflammatory disorders *(Reuter et al., 2010; Birben et al., 2012)*. The presence of substantial amounts of phenolic compounds and flavonoids, together with measurable radical-scavenging activity, provides a plausible scientific basis for several traditional uses of guava leaves, such as the management of diabetes, diarrhoea, inflammation and wounds, and is consistent with the antidiabetic, anti-inflammatory, hepatoprotective and anticancer effects reported for guava leaf extracts *(Oh et al., 2005; Dutta & Das, 2010; Ashraf et al., 2016; Díaz-de-Cerio et al., 2017)*. However, *in vitro* antioxidant activity does not necessarily translate into *in vivo* efficacy, because the absorption, metabolism and bioavailability of polyphenols are limited and complex. Further biological studies are therefore needed before therapeutic claims can be made.

5.8 Limitations of the Study

The present study has several limitations that should be considered when interpreting the results:

- Only one solvent (98% ethanol) and one extraction method (cold maceration) were used; hydroethanolic solvents or modern techniques such as ultrasound-assisted extraction might yield extracts with different phenolic contents and activities.
- A relatively low powder-to-solvent ratio (400 g of powder in 800 mL of ethanol, 1:2 w/v) and a single maceration were used; a larger volume of solvent or repeated extraction (re-maceration) of the residue might have increased the extraction yield (5.06% w/w).
- Antioxidant activity was evaluated using a single radical-scavenging assay (DPPH); other assays such as ABTS, FRAP, nitric oxide and hydroxyl radical scavenging, and lipid peroxidation inhibition were not performed.
- The DPPH and TPC assays were performed with single readings at each concentration, and the ascorbic acid and extract assays were not replicated on different days; therefore, measures of variability for these assays are limited.

- Some extract absorbances in the TPC and TFC assays fell slightly below the lowest calibration standard, and no sample blank was used to correct for the intrinsic colour of the extract at 517 and 415 nm.
- The Folin–Ciocalteu and aluminium chloride methods are non-specific colorimetric assays; individual phenolic and flavonoid compounds were not identified or quantified by chromatographic techniques such as HPLC or LC–MS.
- Leaves were collected from a single location and at a single time point, so the effects of variety, leaf age and season could not be assessed.
- The study was limited to *in vitro* assays; cell-based, animal and toxicological studies were beyond the scope of this undergraduate project.

Despite these limitations, the study provides useful preliminary evidence of the antioxidant potential of guava leaves and establishes a foundation for more detailed pharmacological research.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The present study was undertaken to evaluate the *in vitro* antioxidant activity and the total phenolic and total flavonoid contents of the ethanolic leaf extract of *Psidium guajava* L., prepared by cold maceration of shade-dried leaves in 98% ethanol.

The DPPH assay demonstrated that the extract possesses concentration-dependent free radical scavenging activity, reaching 56.32% inhibition at 200 µg/mL, with an IC_{50} value of 169.04 µg/mL compared with 13.06 µg/mL for the standard ascorbic acid. The Folin–Ciocalteu assay revealed a high total phenolic content of 471.17 ± 50.40 mg GAE/g of dry extract, and the aluminium chloride assay showed a total flavonoid content of 132.01 ± 11.17 mg QE/g of dry extract. The calibration curves of gallic acid (R^2 = 0.9943) and quercetin (R^2 = 0.9944) showed good linearity, supporting the reliability of the measurements.

The radical-scavenging activity of the extract increased in parallel with its phenolic and flavonoid content, suggesting that these compounds are important contributors to its antioxidant effect. Although the extract was less potent than pure ascorbic acid, as expected for a crude plant extract, the findings indicate that guava leaves, an abundant and inexpensive agricultural by-product in Bangladesh, are a rich source of natural phenolic antioxidants. These results provide scientific support for the traditional medicinal use of guava leaves and justify further investigation of their potential in the prevention and management of oxidative stress-related disorders. At the same time, antioxidant activity *in vitro* alone cannot confirm therapeutic efficacy, and further studies are required.

6.2 Recommendations

On the basis of the findings and limitations of the present study, the following recommendations are made for future work:

1. Extraction should be compared using solvents of different polarity (water, 50–70% ethanol, methanol, acetone and ethyl acetate) and techniques such as ultrasound-assisted extraction, in order to identify the conditions that maximise phenolic yield and antioxidant activity.

2. Additional antioxidant assays, such as ABTS, FRAP, nitric oxide, superoxide and hydroxyl radical scavenging, and lipid peroxidation inhibition, should be performed to obtain a more comprehensive antioxidant profile.
3. All assays should be performed in at least triplicate on independent occasions, with appropriate sample blanks, and results should be analysed statistically (e.g., ANOVA).
4. Chromatographic and spectroscopic techniques (HPLC, LC–MS, GC–MS) should be used to identify and quantify individual phenolic compounds and flavonoids, such as quercetin glycosides, catechins, gallic and ellagic acids and ellagitannins.
5. Bioassay-guided fractionation should be carried out to isolate and characterise the most active antioxidant constituents.
6. The effects of guava variety, leaf age, season and geographical location in Bangladesh on the phenolic content and antioxidant activity of the leaves should be investigated.
7. Cell-based studies (e.g., cellular antioxidant activity and cytotoxicity against cancer cell lines) and *in vivo* animal studies should be performed to evaluate biological efficacy, together with acute and sub-chronic toxicity studies to establish safety.
8. The stability of the extract and its phenolic constituents under different storage conditions should be evaluated with a view to developing standardised formulations.

6.3 Potential Applications in the Food and Pharmaceutical Industries

Because guava leaves are abundant, inexpensive and rich in phenolic antioxidants, they have considerable potential for value-added applications *(Lourenço et al., 2019; Kumar et al., 2021)*:

- **Functional foods and beverages:** guava leaf tea and extracts can be used as antioxidant-rich functional ingredients; guava leaf tea is already consumed in East Asia for blood glucose management *(Deguchi & Miyazaki, 2010)*.
- **Natural food preservatives:** phenolic-rich extracts may replace or reduce the use of synthetic antioxidants such as BHA and BHT to retard lipid oxidation in foods *(Lourenço et al., 2019)*.

- **Nutraceuticals and herbal medicines:** standardised extracts could be developed as dietary supplements for supporting antioxidant defence and glycaemic control.
- **Oral care products:** the antimicrobial and anti-inflammatory properties of guava leaves support their use in mouthwashes, toothpastes and gum-care formulations *(Morais-Braga et al., 2016)*.
- **Cosmetics and dermatological products:** antioxidant and tyrosinase-inhibitory activities make guava leaf extracts attractive ingredients for anti-ageing and skin-care products *(You et al., 2011)*.

Such applications could turn a largely discarded agricultural by-product into a valuable resource, benefiting both the pharmaceutical sector and guava growers in Bangladesh.

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