EXTRACTION OF BIOACTIVE COMPOUNDS FROM GUAVA LEAVES

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CERTIFICATION

This is to certify that this project work was carried out and compiled by I, **JOSEPH EMMANUEL** with Matriculation number **ENG1603783** of the Department of Chemical Engineering, Faculty of Engineering, University of Benin, Benin City, Edo State, Nigeria.

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DEDICATION

I want to dedicate this project work to God Almighty, whose grace, wisdom, and strength made this work possible. To Him be all the glory, honor, and praise.

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ABSTRACT

This study aims to optimize the extraction of bioactive compounds from guava leaves using the Soxhlet extraction method to investigate the impact of key variables such as mass of the solvent, temperature and extraction time (hours) on the bioactive extraction yield, to characterize the extracted bioactive compounds to identify key functional groups, and to optimize the bioactive yield.

The study employed a central composite design (CCD), with 19 experimental runs where Response Surface Method (RSM) was utilized to optimize extraction conditions, evaluating the effects of mass (1.00-10.00 g), extraction time (30-300 min), and temperature (50-90°C). ANOVA and quadratic regression models assessed the influence of these variables on the yields of terpenoids and flavonoids. The qualitative and quantitative analysis of extracted compounds was conducted using colorimetric chemical tests and FTIR spectroscopy. Statistical validation included model significance testing (p-values), R^2, adjusted R^2, predicted R^2, and adequate precision.

The qualitative analysis of guava leaf extract identified flavonoids (yellow), terpenoids (reddish-brown), saponins (froth), alkaloids (reddish-brown precipitate), and tannins (greenish-black). Quantitative results showed the highest percentages in flavonoids (15%) and terpenoids (16%), followed by saponins (2%), alkaloids (1.75%), and tannins (0.183%). Extraction efficiency was highest at intermediate conditions, with significant quadratic effects observed for all three independent variables. The regression models yields for the two major extract, terpenoid and flavonoid, demonstrated high accuracy with R^2 is 0.7915 for terpenoid and R^2 = 0.8957 for flavonoid, with ANOVA confirming model significance (F-value = 0.17, p = 0.9585) and (F-value = 8.59, p = 0.0019) for terpenoid and flavonoid respectively. Also, the extraction yield was significantly affected by mass, time, and temperature. Terpenoid yield declined beyond 55 g and 165 min due to solvent saturation, while flavonoids degraded above 70°C. Optimal conditions enhanced solubilization and diffusion, but excessive parameters caused thermal degradation, volatilization, poor solvent penetration, and reduced extraction efficiency. These findings support guava leaves as a rich source of bioactive compounds with antioxidant, anti-

inflammatory, and antimicrobial properties, valuable for pharmaceutical and nutraceutical applications.

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CHAPTER ONE

INTRODUCTION

1.1. BACKGROUND OF STUDY

The guava (*Psidium guajava* L.), a member of the *Mytaceaceae* family, is an evergreen shrub or small arbor with a wide range of habitats (Kong et al., 2015), which is grown for its many nutritional and therapeutic uses and it is special and traditional (L. Wang, Wu, et al., 2017). It is considered an apple of the tropics and a poor man's fruit (Luo et al., 2018), and it is typically found in tropical and subtropical regions, particularly in South Africa, Africa, and Asia (Li et al., 2018). The guava fruit is made up of 13.2% carbs, 0.53% lipids, and 0.88% proteins in an environment that is high in water (84.9%) (Flores et al., 2015). Different tree parts, including the fruit, leaves, shoots, and bark, have long been used to treat a wide range of illnesses, including diabetes, vertigo, skin conditions, jaundice, mental disorders, and dysentery (K. Kumar et al., 2021). The dark green, dry leaves and lush branches of the guava tree are known as guava leaves (*Psidii guajava folium*) (Jiang et al., 2020), and they have been utilized in traditional medicine and have been shown to contain a variety of bioactive substances, including guaijaverin, hyperoside, peltatoside, gallic acid, vescalagin, catechin, and iso-quercetin (Flores et al., 2015). According to Jiang et al. (2020), guava leaf extract has been traditionally used to treat a wide range of illnesses, including antispasmodic, mellitus, sedative, anti-inflammatory, antidiarrheic, antihypertensive, anti-obesity, and antidiabetic characteristics (Jiang et al., 2020). There are only a few papers that confirm the possibility of extracting essential oils from guava leaves, including triterpenoids (0.45 –7.05 mg/g), flavonoids (2.06 –2.61 mg/g), tannins (1.72 – 2.35 mg/g), and carotenoids (5.2 – 11.9 × 10 − 3 mg/g) (Hassan et al., 2021). Due to their anticough effect, guava

leaf extracts have been utilized to treat coughs (Rakmai et al., 2018). Also, it has been determined that the guava leaf's aqueous-chloroform-methanol extract exhibits a strong inhibitory effect against bacteria, including *S. Aureus, Bacillus, and Salmonella* (Waresindo et al., 2021), and human blood glucose levels can be effectively maintained by the phenolic extract of guava leaves (Zhu et al., 2020).

Extraction is an essential procedure that guarantees the necessary release of essential components from the plant matrix into the extract media (Ciric et al., 2020). However, there are several problems with the traditional ways of extracting bioactive chemicals from guava leaves, including maceration, hydro distillation, and hot water extraction. Incomplete solubility of bioactive chemicals during maceration, a time-consuming process that entails soaking plant material in a solvent for a prolonged duration, frequently leads to low extraction efficiency (Mohammadpour et al., 2019), and significant energy input is required for hydro distillation, which might reduce the efficacy of heat-sensitive chemicals due to thermal degradation (Jobson, 2014). Although hot water extraction is easier, there is a chance that thermolabile bioactive chemicals will be degraded, which could result in reduced yields and affected quality (Plaza & Turner, 2015). Environmental and safety considerations are further heightened by the fact that these procedures typically employ substantial quantities of solvents (Mohammadpour et al., 2019). These techniques are less efficient and less sustainable because of the extended periods of extraction and the high energy expenditures linked with them and the overall effectiveness of bioactive chemical recovery is further reduced when undesirable molecules are co-extracted with the compounds being purified (Sampath Kumar et al., 2021a). Also, ultrasound extraction of bioactive compounds from guava leaves faces challenges like optimizing process parameters, potential degradation of thermolabile compounds, high equipment costs, and ensuring uniform

cavitation. These factors can affect the efficiency and yield of bioactive compounds during extraction (Wani & Uppaluri, 2022). More effective and environmentally friendly extraction processes are needed to maximize the yield and quality of bioactive chemicals from guava leaves, as these constraints are brought to light.

For many years, people have extracted herbal plants using the Soxhlet extractor. Soxhlet extraction is a technique used in extraction processes to extract compounds whose solubility is low in a solvent and whose impurity is insoluble in that solvent (Nik Mat Daud et al., 2015). The features of the soxhlet extract include being environmentally friendly, having a lower running cost, and being simple to control and manipulate (Nik Mat Daud et al., 2015). To find the ideal operating conditions for extracting the nonpolar chemicals from guava leaves, the extraction parameters were improved using response surface methodology (RSM) (Mohammadpour et al., 2019). The extraction of bioactive chemicals from guava leaves has been studied, however, there is a noticeable lack of research on this herb in Nigeria, particularly when it comes to optimizing extraction procedures. Existing research has mostly been undertaken in other regions, researching alternative solvents and procedures to maximize the yield and efficacy of these chemicals (Sampath Kumar et al., 2021a). The extraction efficiency and bioactive chemical profiles may be impacted by the unique soil types, ambient circumstances, and guava cultivars found in Nigeria. Therefore, local research is necessary to optimize extraction parameters such as solvent choice, temperature, and extraction duration specific to the Nigerian setting. Additionally, understanding the particular bioactive profiles of Nigerian guava leaves could lead to more effective utilization in medicinal and nutritional applications (Díaz-de-Cerio, Verardo, et al., 2017). Given the potential health advantages and economic value of guava leaves, concerted

research in Nigeria might considerably increase the local herbal medicine sector and promote the use of indigenous resources for health and wellbeing.

1.2. STATEMENT OF PROBLEM

While helpful in different applications, Guava (*Psidium guajava*) leaves pose environmental issues in Nigeria. One notable issue is their possible invasiveness in particular locations, harming native ecosystems due to their allelopathic impacts on other plant species. Also, inappropriate disposal of guava leaf waste has led to environmental pollution and contamination.

Conventional techniques for extracting bioactive chemicals from guava leaves, other than Soxhlet extraction, confront many problems. Maceration is time-consuming and typically results in partial extraction, giving lesser levels of bioactive chemicals. Hydrodistillation demands significant energy for boiling and steam distillation, leading to high energy consumption and probable thermal destruction of heat-sensitive chemicals, therefore lowering their quality and yield. Supercritical fluid extraction, although effective, is expensive due to the high cost of equipment and operational expenses, and it demands exact control of temperature and pressure, which can be tough to maintain. Ultrasound-assisted extraction (UAE) shows potential but has scaling concerns, making it difficult to apply on an industrial scale, and the initial investment in ultrasound equipment can be prohibitive. These constraints underscore the necessity for developing more efficient and cost-effective extraction technologies.

1.3. AIM AND OBJECTIVES

This study aims to extract bioactive compounds from guava leaves using Soxhlet extraction methods.

The study aims to achieve the following objectives:

(i). Preparation of the powdered guava leave samples.

(ii). Optimizing the bioactive extraction using the Response Surface Method (RSM) with the Soxhlet extraction method.

(iii). Investigating the impact of variables such as mass of extract (g), temperature (°C), and time duration of extraction (minutes) on the bioactive extraction yield.

(iv). Characterization of the extracted bioactive compounds.

1.4. SCOPE OF STUDY

This study encompasses both fieldwork and experimental investigations within its scope.

This study will cover the following:

(i). Collection of guava leaves from the University of Benin, Benin City, Nigeria, and its environs.

(v). Utilizing extraction variables such as mass of extract (g), temperature (°C), and time duration of extraction (minutes) on the bioactive extraction yield.

(ii). Characterization of the extracted Bioactive compounds.

1.5. RELEVANCE OF STUDY

This study presents sustainable strategies to address the issue of environmental pollution and contamination due to improper disposal of guava leaves also, it provides a better alternative for bioactive extraction by utilizing the Soxhlet extraction method which is effective for extracting bioactive compounds from guava leaves, including polyphenols, flavonoids, and essential oils. It efficiently isolates phytochemicals and ensures maximum yield. This method is ideal for research and industrial applications, handling large quantities of plant material at low cost.

CHAPTER TWO

LITERATURE REVIEW

2.1. GUAVA

Guava (*Psidium guajava*) is an evergreen bush or little tree in the family Myrtaceae developed for its consumable natural products. Guava (*Psidium guajava* Linn.) is a fruit that is well- known for its culinary and nutritious qualities all over the world (Etim et al., 2021). The fruit is also as "The fruit of the poor guy" or "Tropical apple". Guava is commonly grown in common tropical and subtropical regions (Rani & David, 2021). Guava (*Psidium guajava* Linn.) is a tropical American fruit that was first introduced to India in the early seventeenth century. Guava is grown in India, Mexico, Brazil, Cuba, Venezuela, Australia, South Africa, Thailand, Malaysia, Indonesia, China, Sri Lanka, the Philippines, Bangladesh, Myanmar, the Dominican Republic, the United States, and Haiti (Sharma & Borah, 2021). Guava stands fifth in production among India's most important fruit crops and can be grown throughout the country (Kumar et al., 2020). Depending on the species, guava fruits are typically 4 to 12 centimeters (1.6 to 4.7 in) long, round, or oval. The fruit is initially green in color, but as it ripens, it turns yellow (Kafle et al., 2018). The fruit contains approximately 80 percent moisture and 20 percent dry matter including 1 percent ash, 0.7 percent fat and 1.5 percent protein (Upadhyay et al., 2019).

Guava fruit is often consumed fresh as a dessert fruit or processed as puree, juice, concentrate, jam, jelly, cheese, toffee, fruit flakes, squash, syrup, nectar, powder, wine, vinegar, ready-to-eat snacks, beverages, and dried canned items (Sinha et al., 2017). The root, bark, leaves, and fruit of the plant have been shown to have pharmacological effects (Seshadri et al., 2020) and are used to cure a range of diseases. malaria, gastroenteritis, vomiting, looseness of the bowels, diarrhea,

sores, ulcers, toothache, sore throat, swollen gums, and a host of other symptoms have also been managed with various portions of the plants throughout herbal therapy. This plant has also been utilized to treat life-threatening conditions including diabetes, hypertension, and obesity. Guava fruit comes in two kinds of white and pink interior Interspersed with small firm seeds (Rani & David, 2021). White guava has a sweeter flavor and is more extensively cultivated, whereas pink guava is considered a delicacy. The fruits are circular to ovoid, meaty, yellow, and roughly 5cm in diameter, with a pink or white edible mesocarp containing little round seeds (Sharma & Borah, 2021).

Figure 2.1: Guava

Guava seeds make up between 6-12 percent of the overall weight of the fruit. The seeds are spherical in shape and pale yellowish brown in color, with 16 percent oil, 7.6 percent protein, and 61.4 percent crude fiber content. Guava seeds, have the ability to become a source of oil that can be utilized in culinary items and as a nutritional supplement (Raihana et al., 2015). The leaves are opposite, rectangular, three to seven inches long, and have pronounced veins on the

underside. Guava leaf is extensively used to treat diarrhea, gastroenteritis, and other digestive disorders, while the fruit of the guava has been utilized to boost platelets in dengue fever patients (Laily et al., 2015). The guava bark is thin and has green streaks on it. It is quite uncomplicated to dispense with it in long straps. It includes a significant volume of antimicrobial and antibacterial compounds (Naseer et al., 2018).

Guava is a highly rich source of ascorbic acid (vitamin C) and contains other nutraceutical components, including vitamin A (beta-carotene), vitamin B1 (thiamine), (B2) riboflavin, niacin and pantothenic acid (Anand et al., 2020). Moreover, it additionally contains a large quantity of phosphorus, calcium, iron, potassium, and sodium. The primary constituents of guava are citric acid and acetic acid (Palachum et al., 2020). Guava's dietary relevance is strengthened by the presence of antioxidant pigments such as carotenoids and polyphenols. These varied bioactive nutrients have a vital part in traditional therapy for various lifestyle issues, such as diabetes (type 2) and obesity (Upadhyay et al., 2019). α-pinene, β-pinene, limonene, menthol, β-sitosterol, cineol, quercetin are bioactive chemicals with diverse pharmacological action (Ngbolua, 2018).

2.2. Global Cultivation of Guava

Guava (*Psidium guajava*) is a widely cultivated tropical fruit, known for its nutritional value and adaptability to various climates (Sharma & Borah, 2021). Originating in tropical America, guava trees are now grown in many tropical and subtropical regions worldwide, including parts of Asia, Africa, and the Americas. It is cultivated in both tropical and subtropical regions up to an altitude of 1500 meters above mean sea level (Fischer & Melgarejo, 2021). It can endure high temperatures and drought, particularly during the summer months in northern India being the largest producer of guava, leading the world with an annual production of approximately 17.65 million metric tons. The states of Uttar Pradesh, Punjab, and Tamil Nadu are particularly

renowned for guava cultivation. Other major producers include China and Thailand, which contributed significantly to global guava production, totaling around 55 million metric tons in 2019, and it is also cultivated in smaller quantities in countries like Nigeria, where it is an important part of traditional agriculture and local diets (Sharma & Borah, 2021).

Predominantly grown in tropical areas, guava can withstand temperatures ranging from 15°C to 45°C (59°F to 113°F). Optimal growth occurs between 23°C and 28°C (73°F to 82°F), although mature trees can handle short periods at -3°C to -2°C (27°F to 28°F) (Angulo López et al., 2021). Temperatures below 15°C (60°F) may halt fruit production. However, guava is susceptible to severe frost, which can damage young plants. An annual rainfall of about 100 cm during the rainy season (July to September) is sufficient, though rain during harvest can affect fruit quality. Guava thrives in various soil types, including sandy, rocky, and loamy soils, with a preferred pH range of 4.5 to 7 but can tolerate alkaline soils up to a pH of 8.5 (Birdi et al., 2020). It is more drought-resistant than most tropical fruits and can survive extended dry periods by pausing vegetative growth until conditions improve. High-quality guavas are often grown in river basins, but the crop is susceptible to waterlogging (Sehrawat et al., 2014). The rainy season, particularly in June and July, is ideal for planting layers and seedlings. India leads global guava production, with approximately 200,640 hectares dedicated to guava cultivation. Various guava cultivars are grown across the country, with a reported productivity of 15.3 MT/ha. Uttar Pradesh (UP) was the top guava-producing state, with 928.44 tonnes produced in 2017-2018.

2.3. Taxonomy of Guava

The guava plant, scientifically known as *Psidium guajava L.*, is a member of the Myrtaceae family. Within the *Psidium* genus, which consists of roughly 150 species, *Psidium guajava* stands out as the most significant fruit. It is believed that guava originated in a region spanning

from southern Mexico through Central America. Guava is now well-known and cultivated across the globe (Angulo-López et al., 2021).

Kingdom: Plantae

Order: Myrtales

Family: Myrtaceae

Subfamily: Myrtoideae

Genus: Psidium

Species: Guajava

Binomial name: *Psidium guajava Linn.*

2.4. Nutritional Value of Guava

This widely consumed fruit is packed with essential nutrients, making it a valuable part of a balanced diet. It provides a good amount of carbohydrates, proteins, fats, and minerals, which can be beneficial in preventing malnutrition (Youssef & Ibrahim, 2016). The USDA's Food Data Central highlights guava as a notable source of protein (2.3%), carbohydrates (12.16%), and dietary fiber (4.8%). Additionally, guava offers a solid supply of calcium at 17.63 mg per 100 g, and it is exceptionally rich in ascorbic acid, providing 241.86 mg/100 g, which, along with other fruits, enhances its potential for use in food products (Krishi Vidyapeeth et al., 2020). Guava is also abundant in lutein, zeaxanthin, lycopene, flavonoids, fructose, and carotenoids. The fruit contains significant levels of calcium, phosphorus, iron, and various vitamins, including niacin, pantothenic acid, thiamin, riboflavin, vitamin A, and vitamin E (Sharma & Borah, 2021). Its

polyphenolic compounds and carotenoids contribute to its strong antioxidant properties, ranking it among the fruits with the highest antioxidant content (Omayio et al., 2019). Guava also contains essential oils, phenols, triterpenes, saponins, flavonoids, lectins, fiber, pectin, and fatty acids. The fruit is rich in polyphenols and glycoside esters like caffeic, coumaric, ferulic, cinnamic, ellagic, and rosmarinic acids (Medina & Valdés-Infante Herrero, 2015). Flavonoids such as myricetin, naringenin, epicatechin, quercetin, rutin, and apigenin are also present (Anand et al., 2020). Key components of guava include α-Pinene, β-caryophyllene, (Z)-3-hexenal, and α-humulene, as well as various carbonyls and esters like 3-hydroxy-2butanone, benzaldehyde, ethyl hexanoate, (Z)-3-hexenyl acetate, hexyl butanoate, and ethyl octanoate. Guava leaves are rich in various chemical compounds, including α-pinene, β-pinene, limonene, menthol, caryophyllene, β-bisabolene, farnesene, humulene, selinene, cardinene, and curcumene. They also contain malic acids, β-copanene, β-sitosterol, cineol, quercetin, tannin, guajavolide, guavenoic acid, resin, triterpenes like oleanolic acid, triterpenoids, prenol, dihydrobenzophenanthridine, and cryptonine. Guava seeds are notable for their bioactive components such as polyphenols, tocopherols, and phytosterols, which offer significant health benefits. The seeds have a protein content of 9.73% dry matter, including 15 amino acids, predominantly arginine, glutamic acid, aspartic acid, glycine, and leucine. The primary fatty acids in guava seed oil are linoleic (60.0%), palmitic (14.8%), oleic (12.5%), stearic (9.08%), and arachidic (1.31%). Due to its high linoleic acid content, guava seed oil is a valuable source of omega-6 essential fatty acids, and it also provides a significant amount of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Guava seeds contain proteins, oils, phenolic compounds, flavonol glycosides, starch, and quercetin-3-O-D-(2"-Ogalloyglucoside)-4'-O-vinylpropionate. The twigs of *Psidium guajava* are rich in minerals like calcium, magnesium, phosphorus, potassium, sodium, and trace elements such as fluoride, copper, iron, zinc,

manganese, and lead. Additionally, they contain flavonoids, sesquiterpene alcohols, and triterpene acids. The bark has 12–30% tannin content, with some sources reporting up to 27.4% tannin or polyphenols, resin, and calcium oxalate crystals. Tannin is also present in the roots, which additionally contain leukocyanidins, gallic acid, and sterols (Sharma & Borah, 2021).

2.5. Utilization of Different Part of Guava

Guava is transformed into numerous processed products, such as beverages, syrups, ice creams, jams, jellies, toffees, juices, as well as dried and canned goods. Moreover, various parts of the guava plant are employed in different pharmacological uses (Sharma & Borah, 2021).

2.5.1. Food Utilization

The guava offers vast commercial opportunities because it is easy to grow, highly nutritious, and can be processed into a wide range of products. Guava can be used to make products like jam, juice, pulp, jellies, chocolate, wine, guava powder (often used in yogurt preparation), and spray-dried guava extracts, which are rich in antioxidants (Kanwal et al., 2016).

2.5.2. Guava Jams and Jellies

Jam is a thick, semi-solid product made by cooking soft fruit tissue with sugar. It is a moderately moist food produced by boiling fruit pulp with sugar (sucrose), pectin, acid, and other additives like color and flavoring agents until it reaches a consistency that can hold the fruit tissue in place (Rahman et al., 2018). The jam typically reaches a Brix level of 65 to 68 degrees, after which it is hot-filled into sterilized glass jars. Guava jelly is made from slightly ripe guava. Jelly is a semi-solid product prepared by boiling clear, strained fruit juice, free of pulp, with added sugar, citric acid, and pectin. It should have at least 65% total soluble solids and at least 45% fruit content (V. Kumar et al., 2020). The fruit is cut into small pieces and cooked at low temperatures for about 45 minutes, with an equal amount of water. The juice is then extracted by filtering

through a sieve or muslin cloth (Kuchi et al., 2014). Sugar is added to the extracted juice and boiled until it reaches 105°C or a layer forms when a small portion is cooled on a spoon. The amount of sugar used depends on the pectin content of the juice, ranging from 0.5 kg of sugar per kg of juice for pectin-rich juice to 0.75 kg for low-pectin juice. Finally, the jelly is hot-filled into clean and sterilized containers (Swer et al., 2018).

2.5.3. Guava Leathers

Guava leather is made by dehydrating fruit purée into a thin, leathery sheet. It can be enjoyed as a sweet treat or used as an ingredient in sauces. There is limited information available in tropical regions regarding the chemical and sensory properties of guava leather. This product is noted for having higher levels of protein and fat. Additionally, the ash content in guava leather (2.87%) is slightly higher compared to pawpaw leather (2.67%) (Kanwal et al., 2016).

2.5.3. Guava Shrikhand

The rising demand for low-fat products that mitigate the risk of chronic diseases has led to the creation of probiotic low-fat foods like shrikhand, a light, sweet-tart fermented dairy product. It is a well-loved treat in Gujarat, Maharashtra, and Karnataka. The process of making shrikhand starts with heating skim milk, then cooling it to 30°C in a batch pasteurizer. A lactic acid bacteria (LAB) starter culture is added, and the mixture is thoroughly blended. During incubation, the pre-sterilized storage vat is maintained at 37°C for 8 to 12 hours. Once the curd has set, it is strained through a clean, damp muslin cloth into another container. Sugar and guava powder are then incorporated into the chakka, mixing until smooth, either by hand or mechanically. The final product is typically packaged in polystyrene cups and stored in refrigerated conditions (Sharma & Borah, 2021).

2.5.4. Guava Juice and Nectars

Guava juice is made from either fresh guava fruits or guava pulp. The juice is extracted by pressing the guava fruit with a hydraulic filter press or by diluting the pulp with water and then filtering it. Since the juice is typically not clear, pectic enzymes are used to improve its clarity and ease of processing. Research by Imungi identified that the optimal conditions for extracting guava juice using proteolytic enzymes from Kenyan guavas involve using 400 ppm of the enzyme at a temperature of 45-50°C for 90 minutes. For nectars, water is added to guava pulp or fresh juice. Sweeteners or sugar may be added, but the final product must have a minimum of 8.5° Brix, contain at least 25% guava puree or juice, and maintain acidity at 0.15% with a pH between 3.4 and 4.8 (Omayio et al., 2019).

2.5.5. Guava Wine

Guava wine (*Psidium guajava* L.) is produced through an anaerobic fermentation process, where yeast converts the sugars present in the fruit into alcohol and carbon dioxide (Sharma & Borah, 2021).

2.5.6. Guava Leaf Tea

Hot water extraction of *Psidium guajava* leaves produced a 14% yield of guava leaf extract. To summarize, 100 g of dried leaves were combined with 2 liters of distilled water and simmered at 80°C for 30 minutes. Afterward, the solution was filtered through four layers of gauze to remove the leaves, and the extract was freeze-dried (Anand et al., 2020).

2.5.7. Guava Leaf Powder

Guava leaves were gathered and finely chopped by hand. The material was then rinsed several times with regular tap water and once with distilled water to remove dust and soluble contaminants. After washing, the leaves were left in a clean, dry area to air-dry, followed by

drying in an electric oven at 50°C for 24 hours. Once dried, the guava biomass was cooled in a desiccator, ground into a powder, and stored in a moisture-free environment for future use (Ponnuchamy et al., 2020).

2.5.8. Guava Seed Powder

Initially, the seeds were collected and dried using an air circulation oven (Tecnal, model TE-394/L) at 60°C for about 16 hours. Once the drying process was complete, the seeds were ground with a household blender (Walita) to produce a powder. This powder was then stored in sealed polyethylene containers (Silva et al., 2014).

2.5.9. Guava Seed Powder Fortified Biscuits (GSPFB)

Using response surface methodology (RSM), the proportions of guava seed powder, wheat flour, and sugar were optimized. A mixture consisting of 17.65 grams of guava seed powder, 62 grams of wheat flour, and 20 grams of sugar was prepared. The recipe also included 25 grams of butter, 1 gram of baking powder, and 2 milliliters of vanilla extract. The oven was preheated to 180°C. The dough was rolled out and shaped into rounds. The shaped dough was placed on a food-grade steel mesh on an oven tray and baked at 180°C for 25 minutes. After baking, the biscuits were allowed to cool at room temperature for 30 minutes. Control biscuits, made without guava seed powder, were also prepared. Each biscuit formulation was produced in triplicate. The biscuit-making process followed the guidelines outlined by the AACC (2000). Once cooled, the biscuits were packed in low-density polyethylene pouches and sealed for further analysis (Sharma & Borah, 2021).

2.5.10. Guava Pomace

Guava pomace, which constitutes up to 15% of the original fruit, is the by-product left after juicing guava fruits (Denny et al, 2013). To process this pomace, a cabinet tray dryer was

employed, capable of maintaining a drying temperature between 20 and 150°C. Given its high moisture content, the guava pomace was dried at 65°C, a temperature recommended in earlier research for drying similar by-products such as carrot pomace.

2.6. PHARMACOLOGICAL UTILIZATION OF THE DIFFERENT PARTS OF GUAVA.

Guava has been explored for its therapeutic applications by various experts and is believed to hold promise for treating a wide range of diseases globally. Its efficacy has been supported by ethnopharmacological studies, research institutions, and preliminary clinical trials. Additionally, safety assessments of guava's roots, bark, leaves, fruit, flowers, and seeds have confirmed their safety for both oral and topical therapeutic use when prepared in proper formulations (Gupta et al., 2020).

2.6.1. Antioxidant Activity

Recent research indicates that *Psidium guajava* is a significant source of phytochemical antioxidants. Guava is highly regarded for its antioxidant properties and is a rich source of vitamin C. The antioxidant effects of guava are primarily due to the polyphenols present in its leaves. Extracts from guava leaves and essential oils from the stem and bark have been shown to neutralize free hydrogen peroxide and superoxide anion radicals, while also preventing the formation of hydroxyl radicals. Key compounds contributing to these antioxidant properties include quercetin, carotenoids, vitamin C, and various polyphenols (Dakappa et al, 2013). Quercetin, along with quercetin-3-O-glucopyranoside and morin, can be extracted from the leaves and is noted for its potent antioxidant activity, with quercetin being considered the most effective antioxidant in guava leaves.

2.6.2. Anti-diabetic

Psidium guajava has been linked to blood glucose regulation. Studies indicate that guava fruit extract aids in weight loss and blood sugar management in diabetic patients. When administered at doses of 125 and 250 mg/kg to STZ-induced diabetic subjects, guava fruit extract was found to protect pancreatic tissues, particularly insulin-producing beta cells, from oxidative damage, thus preserving insulin production. Additionally, quercetin, kaempferol, and myricetin in the extract were observed to inhibit enzymes like sucrose, maltase, and α-amylase (Ismail Iid et al., 2020).

2.6.3. Anti-inflammatory Activity

Guava extract in ethyl acetate has been found to prevent germ contamination and inhibit thymus production, showing promise as an antiviral agent. It also enhances mRNA expression and may influence the activity of the heme oxygenase-1 protein. This makes guava extract useful as a skin anti-inflammatory agent. Additionally, ethanol-extracted guava inhibits the lipopolysaccharide-induced production of nitric oxide and suppresses the release of E2, further contributing to its anti-inflammatory effects (Jang et al, 2014).

2.6.4. Anticancer Effect

Several studies have demonstrated that *Psidium guajava*, a medicinal plant, has significant effects on human epidermal carcinoma and murine leukemia cells. The LDH release assay, MTT reduction assay, and colony formation assay all confirmed the strong cytotoxicity of guava branch extract (GBA). The extract inhibited the growth of HT-29 cells at a concentration of 250 µg/ml. Additionally, the branch extract induced apoptosis in HT-29 cells, evident by chromatin condensation and cell shrinkage. It also increased cytotoxicity and elevated the sub-G1 phase in HT-29 cells (Lok et al., 2023).

2.6.5. Antiviral Activity

The antiviral activity of guava extracts was tested against the A/Narita/1/2009 strain (a 2009 pandemic strain resistant to amantadine) and demonstrated an IC50 of 0.05 percent. The extracts also inhibited the growth of the A/Yamaguchi/20/06 strain (a sensitive strain) and the A/Kitakyushu/10/06 strain (resistant to oseltamivir). The growth of these strains was significantly suppressed by guava extracts. Guava tea has proven to be effective against influenza viruses and has also been shown to enhance the body's viral resistance (Wang et al., 2014).

2.6.6. Antidiarrhoeal Effect

Quercetin and quercetin-3-arabinoside, extracted from the buds and leaves of *Psidium guajava L.* at a concentration of 1.6 μg/ml, exhibited an effect similar to morphine by inhibiting the release of acetylcholine in the electrically stimulated ileum. This action was accompanied by an initial increase in muscle tone followed by a gradual decrease. Additionally, a methanol extract of guava leaves at 8 μg/ml demonstrated 93.8% inhibition against the simian (SA-11) rotavirus. Moreover, the guava-derived galactose-lectin was found to bind with *Escherichia coli*, a common cause of diarrhea, preventing it from adhering to the intestinal wall and thereby reducing diarrheal inflammation (Sharma & Borah, 2021).

2.6.7. Immunomodulatory Activity

Guava leaf extract has demonstrated the ability to modulate the immune system. Specifically, a decoction of guava leaves can stimulate macrophages to target and eliminate E. coli strains that produce heat-stable toxins, as tested in murine monocyte cell line J774 (Birdi et al., 2014). Additionally, the ethyl acetate fraction from guava leaves has been found to inhibit COX2 expression, reduce cytokine release, and block both degranulation and FceRI-mediated signaling

in mast cells that have been activated by antigens. Moreover, a flavonoid extract from guava leaves has been shown to regulate the activation of the nuclear factor KB in an in vitro model using renal macrophages from Labeo rohita (Daswani et al., 2017).

2.6.8. Antiparasitic Activity

Antiparasitic medications are used to address infections caused by various parasites, including ectoparasites, protozoa, parasitic fungi, and helminths. In laboratory tests, essential oil derived from guava leaves demonstrated effective antiparasitic properties against Toxoplasma gondii. The potential therapeutic effects of this essential oil may include a reduction in free radicals linked to the pathology of toxoplasmosis (Lee et al., 2013)

2.6.9. Wound Healer

The periodontal fibers, including those in the gums and ligaments, are mainly composed of collagen. Fibroblasts, which are prevalent in the connective tissue of the periodontium, play a crucial role in maintaining its structure. Adequate vitamin C is essential for preserving the health of this tissue. Guava, which is high in vitamin C (ascorbic acid), can influence collagen production by regulating procollagen gene expression and affecting fibroblast function and the extracellular matrix. Additionally, a decoction made from guava root bark is used as a mouthwash for sore gums, while a leaf decoction serves as an effective gargle for inflamed and bleeding gums (Ravi & Prasad, 2014).

2.6.10. Antibacterial

Guava extracts exhibit antibacterial properties effective against both Gram-positive and Gram-negative bacteria. Laboratory tests demonstrated that both aqueous and water-soluble methanol extracts from guava leaves and bark have significant antibacterial activity against multidrug-resistant Vibrio cholerae (Sharma & Borah, 2021).

2.6.11. Guava for Cold and Cough

Guava leaves are known to aid in treating colds and coughs due to their high ascorbic acid and iron content. These nutrients help alleviate lung inflammation and excess mucus production while keeping the respiratory system free from pathogens. Both raw guava fruit and a decoction of young leaves are highly effective for these ailments. The astringent qualities of guava help break down mucus, reduce coughing, and minimize mucus accumulation, which keeps the respiratory tract, mouth, and lungs clean of bacteria and inhibits microbial activity. Guava's rich vitamin C content supports its effectiveness in combating colds and coughs caused by infections. In some Indian villages, roasted ripe guava is used as a traditional remedy for severe coughs, colds, and congestion. Additionally, research indicates that a hydro extract from guava leaves significantly reduces coughing caused by capsaicin aerosol within 15 minutes compared to a control group (Kafle et al., 2018).

2.6.12. Hematological Activity

Methanolic extracts from the bark of *Psidium guajava* are effective for boosting blood in anemic individuals and can also be used as a preventive measure. A dosage of 200 mg/kg has been proposed as adequate to promote the required production of blood cells. Although the precise mechanism by which the extract influences these hematological improvements was not specified in the study, it is inferred that the extract directly affects the hematopoietic system (Manekeng et al., 2019).

2.7. BIOACTIVE COMPOUNDS

The definition of bioactive compounds remained ambiguous and unclear for a long time. Very few references describe the term "bioactive". It is composed of two words bio- and -active. In etymology bio- is from the Greek (βίο-) "bios" that means life while -active is derived from the

Latin word “activus” that refers to dynamic, full of energy, with energy, or involved in activity. The term “bioactive” is an alternative term for “biologically active”. Hence, a bioactive compound is simply a substance with biological activity (Guaadaoui et al., 2014).

A plant extract is a substance or an active substance with desirable properties removed from the tissues of a plant, frequently by treating it with a solvent, to be used for a particular purpose. The term “bioactive compounds” is generally referred to as biologically significant chemicals but not established as essential nutrients (Varma, 2016). Bioactive compounds are essential (e.g., vitamins) and non-essential (e.g., polyphenols, alkaloids, etc.) compounds that occur in nature, are part of the food chain, and can affect human health. They are derived from various natural sources such as plants, animals, microorganisms (e.g., fungi) and marine organisms (e.g., lichens) (Swamy & Akhtar, 2019). The amount of bioactive natural products in natural sources is always fairly low (K. Patel et al., n.d.). Plant active compounds are usually contained inside plant matrixes. Active compounds are synthesized in small quantities and different concentrations in all plant organs or parts such as leaves, roots, barks, tubers, woods, gums or oleoresin exudations, fruits, figs, flowers, rhizomes, berries, twigs, as well as the whole plant. Further processes may be required after extraction to purify or isolate the desired compounds.

2.8. GUAVA LEAVES

The dark green, elliptical, oval guava leaf (Psidii guajavae folium; GL) has an obtuse tip. Aside from increasing platelets in dengue fever patients, guava leaves, pulp, and seeds are used to treat certain gastrointestinal and respiratory issues (Laily et al., 2015). Antihypertensive, antiobesity, antidiarrheic, antispasmodic, cough sedative, anti-inflammatory, and antidiabetic are some of the many other common uses for GLs. The function of GL isolates as powerful anticancer, cytotoxic,

and antitumor drugs has been shown in animal models via research (Ashraf et al., 2016; Jiang et al., 2020).

Figure 2.2: Guava Leaves

Fruit pulp is used to increase platelet count in dengue fever treatment, while GLs are often used to treat diarrhea and gastrointestinal problems. Additionally, research on the efficacy of guava leaf extracts in the treatment of diarrhea was conducted (Mazumdar et al., 2015). The antibacterial action of guava leaf extract is mainly due to the flavonoids found in the leaves; specifically, quercetin, the most abundant flavonoid in guava leaves, has potent antidiarrheal effects. One theory for quercetin's antidiarrheal effects is that it relaxes the muscles lining the intestines, making them less likely to contract. Antioxidant food additives and diabetic treatments both have potential in guava leaf polysaccharides (GLPs). Quercetin and other flavonoids, as well as ferulic, caffeic, and gallic acids, are among the distinctive polyphenolic chemicals found in guava leaves that are responsible for their bioactive and medicinal qualities (Farag et al., 2020). These phenolic molecules are known as secondary metabolites which demonstrate substantial antioxidant and immunostimulant properties.

2.8.1. Chemical Composition of Guava Leaves

2.8.1.1. Proximate Composition Guava

Guava leaves (GLs) are a rich source of several health-promoting micro- and macronutrients as well as bioactive chemicals. They include 82.47% moisture, 3.64% ash, 0.62% fat, 18.53% protein, 12.74% carbs, 103 mg ascorbic acid, and 1717 mg gallic acid equivalents (GAE)/g total phenolic components (Shabbir et al., 2020).

2.8.1.2. Polysaccharides

Polysaccharides are polymers that are ubiquitously found in nature. They are made of long polymeric chains, which are formed of monosaccharide molecules. These polysaccharides display different physicochemical, biological, and pharmacological characteristics, such as antioxidant, anti-inflammatory, antidiabetic, immunomodulatory, and anticancer activity (Luo et al., 2019). Guava leaf polysaccharides (GLPs) may be separated using ultrasound-assisted extraction (UAE) (time: 20 min, power: 404 W, temperature: 62 ◦C). These GLPs comprise roughly 9.13% uronic acid and 64.42% total sugars, out of which 2.24% are reducing sugars. GLPs are soluble in water, yet insoluble in organic solvents such ethanol, diethyl ether, ethyl acetate, acetone, and chloroform. Extracted GLP at a concentration of 100 µg/mL showed excellent antioxidant activity with 56.38% and 51.73% 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical- and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation-scavenging ability, respectively (Luo et al., 2018). Similar findings were also reported by Kong et al. (2015). They achieved up to 0.51% GLP employing UAE that displayed strong DPPH•- and •OH-scavenging activity (72–86% and 42.94–58.33%). GLPs may be divided into two groups: unsulfated and sulfated GLPs. Sulfated GLP comprises around 18.58% sulfate content. Sulfated GLP displayed excellent antioxidant activity in terms of DPPH, hydroxyl, and alkyl radical-scavenging activity (0.10, 0.02, and 0.17 IC50, mg/mL, respectively) (Kong et al., 2015).

Studies demonstrated that guava leaf extracts (GLE) substantially decreased the oxidative stress and toxicity generated by hydrogen peroxide in mammalian cell lines (Vero cells) (Kim et al., 2016). GLPs have also been reported to be effective in treating diabetes mellitus symptoms. Acarbose (an antidiabetic medication) is extensively used for the treatment of type 2 diabetes (Luo et al., 2018). It functions as an inhibitor of glycoside hydrolases like α-glucosidase and α-amylase and hence limits fast glucose release from complex carbs (Zhang et al., 2016). This action causes some of the incompletely digested complex carbohydrates to stay in the gut and be transferred to the colon. The intestinal bacteria digest these complex carbohydrate components, causing gastrointestinal disorders such as diarrhea and flatulence. Research found that GLP inhibited α-glucosidase more effectively than acarbose without substantially limiting the α-amylase activity (Z. Zhang et al., 2016). Moreover, it also generated a large decline in fasting blood sugar, total cholesterol, total triglycerides, glycated serum protein, creatinine, and malonaldehyde in diabetic mice without having any serious negative effects (Luo et al., 2019). Therefore, GLP may be utilized as a substitute for acarbose for controlling diabetes mellitus and also as an antioxidant ingredient in meals.

2.8.1.3. Proteins

Guava leaves have 9.73% protein on a dry weight basis (Rahman et al., 2013). Proteins are large biomolecules made of amino acids that function as building blocks of cells. Proteins have a vital function in growth and maintenance, enzyme control, and cell signaling, and also as biocatalysts. Recently, plant-based nutrients have gained promise because of the increased demand for nutritionally abundant meals, notably protein. A considerable effort is currently being undertaken to identify highly sustainable nutritionally dense food sources (Lonnie et al., 2018). Thomas et al. (2017) observed 16.8 mg protein/100g and 8 mg amino acids/100g in guava leaves as calculated

according to Lowry's and ninhydrin techniques, respectively (Thomas et al., 2017). Jassal et al. (2019) noted that guava leaves may be exploited as a new and sustainable food source since they are a high source of proteins, carbs, and dietary fibers (Jassal & Kaushal, 2019).

2.8.1.4. Minerals and Vitamins

Guava leaves are the abundant source of minerals, such as calcium, potassium, sulfur, sodium, iron, boron, magnesium, manganese, and vitamins C and B. The increased concentrations of Mg, Na, S, Mn, and B in GLs makes them a very ideal option for human nutrition and also as an animal feed to combat micronutrient deficiencies (Adrian et al., 2015). Thomas et al. (2017) reported the concentration of minerals such as Ca, P, K, Fe, and Mg as 1660, 360, 1602, 13.50, and 440 mg per 100g of guava leaf dry weight (DW), respectively. The content of vitamins C and B was 103.0 and 14.80 mg per 100g DW, respectively. Consumption of Ca- and P-rich GLs minimizes the incidence of deficiency-related disorders such hypocalcemia, hypophosphatemia, and osteoporosis. The research also found that the content of Ca, P, Mg, Fe, and vitamin B in GLs was greater than that in guava fruit. The increased vitamin C concentration in GLs may aid in enhancing the immune system and preserve the health of blood vessels, whilst vitamin B plays a key role in increasing blood circulation, nerve relaxation, and cognitive function stimulation (Thomas et al., 2017).

2.8.2. Phytochemical Profile

2.8.2.1. Essential Oil Profile

GLs are a rich source of essential oils. The primary ingredient of GL essential oil contains 1,8-cineole and trans-caryophyllene. Ecuadorian GL essential oil exhibited a larger concentration of monoterpenes (limonene and α-pinene) while Tunisian guava leaf oil revealed a higher content of veridiflorol and trans-caryophyllene (Khadhri et al., 2014). Soliman et al. (2016) found a

bigger proportion of monoterpenes, unlike to the previous research, where sesquiterpenes formed the major component in GL essential oil (Soliman et al., 2016). El-Ahmady et al. (2013) found 4 α-selin-7(11)-enol, α-selinene, β-caryophyllene, and β-caryophyllene oxide as the primary components of GL essential oil (El-Ahmady et al., 2013). In another research, sixty-four distinct chemicals were detected in essential oil extracted from GLs using gas chromatography–mass spectrometry (GC–MS). Among these, caryophyllene (24.97%) was discovered to be largely prevalent, which functions as an antioxidant, anticancer, anti-inflammatory, and antibacterial agent. This research reported the content of non-oxygenated sesquiterpenes, oxygenated sesquiterpenes, and monoterpenes as 73.67, 12.94, and 8.55%, respectively (Jassal & Kaushal, 2019).

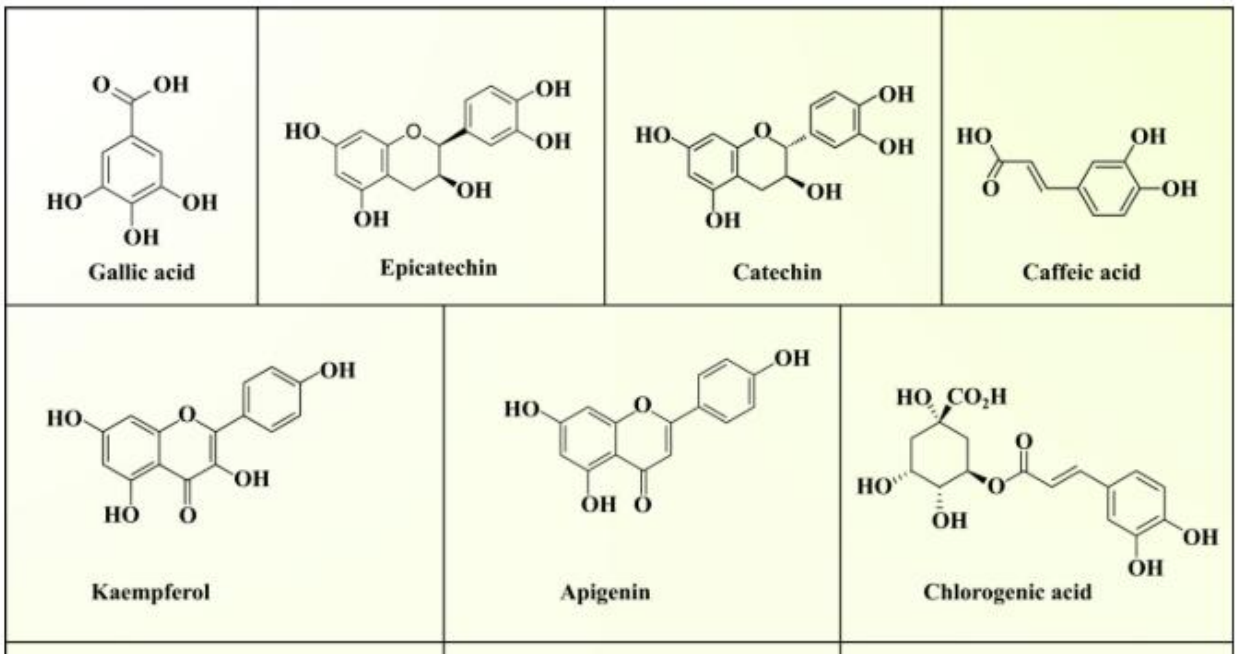
2.8.2.2. Phenolic Compounds

GLs are extremely renowned as a traditional source of medicine in Asian nations owing to their antihyperglycemic function. As discussed in the preceding sections, they include excellent quality bioactive polysaccharides, proteins, lipids, essential oils, vitamins, and minerals. The different secondary metabolites contained in GLs include phenolic acids, flavonoids, triterpenoids, sesquiterpenes, glycosides, alkaloids, and saponins. Phenolic compounds (PCs) serve as important bioactive molecules that give antioxidant and hypoglycemic effects to GLs. Generally, these PCs play a vital part in controlling numerous metabolic and physiological functions in the human body. About seventy-two distinct phenolic compounds have been determined in GLs utilizing high-performance liquid chromatography– diode array detector–quadrupole time-of-flight tandem mass spectrometry (Díaz-de-Cerio et al., 2016). Generally, five quercetin glycosides are found in GLs. The existence of two novel benzophenone galloyl glycosides (guavinosides A and B) and one quercetin galloyl glycoside (guavinoside C) was also

observed (Rasouli et al., 2017). Seventeen varieties of triterpenoids, thirty types of flavonoids, and nineteen types of sesquiterpenoids in GLs have also been identified (Jiang et al., 2020). Moreover, diphenylmethane sesquiterpenoid-diphenylmethane meroterpenoids (psiguadials A and B) and psiguanins A–D (1–4) were also discovered in GLs. Epidemiological studies have shown the effects of polyphenolic chemicals against chronic illnesses, such as diabetes, cancer, and neurological and cardiovascular disorders (Rasouli et al., 2017). Phenolic chemicals influence several physiological processes as cell proliferation, enzymatic activity, cellular redox potential, and signal transduction pathways to fight against chronic illnesses (Luca et al., 2020).

Among phenolic compounds, quercetin is a prominent bioactive phenolic compound in GLs. Diets enriched with bioactive substances have been attracting great attention in recent years owing to their ability to lessen the risk of the development of several chronic illnesses. Seven pure chemicals, quercetin, avicularin, apigenin, guaijaverin, kaempferol, hyperin, and myricetin, were isolated from the ethyl acetate (EtOAc)-soluble GL fraction using Sephadex LH-20 column chromatography with reversed-phase thin layer chromatography (RP-TLC) to monitor separation. Mass spectrometry and nuclear magnetic resonance spectroscopy were employed to elucidate the chemical structures. Wang et al. (2017) extracted and evaluated phenolic compounds from non-fermented guava leaves (NFGLs) and fermented guava leaves (FGLs) using high-performance liquid chromatography coupled to electrospray ionization quadrupole–time-of-flight mass spectrometry (HPLC–TOF–ESI/MS). The authors reported the presence of gallic acid, rutin, chlorogenic acid, avicularin, isoquercitrin, quercitrin, and kaempferol in NFGL and FGL samples. Among these, quercetin, rutin, gallic acid, avicularin, and isoquercitrin represented around 65% of the overall peak area on the chromatogram (Wang, Bei, et al., 2017). Another investigation revealed greater concentrations of catechin (2.25%) and epicatechin (1.45%),

although gallic acid, chlorogenic acid, quercetin, caffeic acid, and epigallocatechin gallate were detected in lower quantities in GL extract (Liu et al., 2014). Additionally, phenolic substances (eugenol and isoeugenol) and al- kaloids (cevadine and emetine) were discovered. Díaz-de-Cerio et al. (2017) improved the extraction of proanthocyanidins, as antidiabetic and antiobesity compounds (Díaz-de-Cerio, Pasini, et al., 2017), from GLs by HPLC–fluorimetric detector (FLD)–ESI–MS and examined their degree of polymerization in various oxidation states. Thus, the phytochemical profile of GL extract displays the existence of various phytochemicals with diverse therapeutic capabilities, indicating its applicability to heal human ailments.



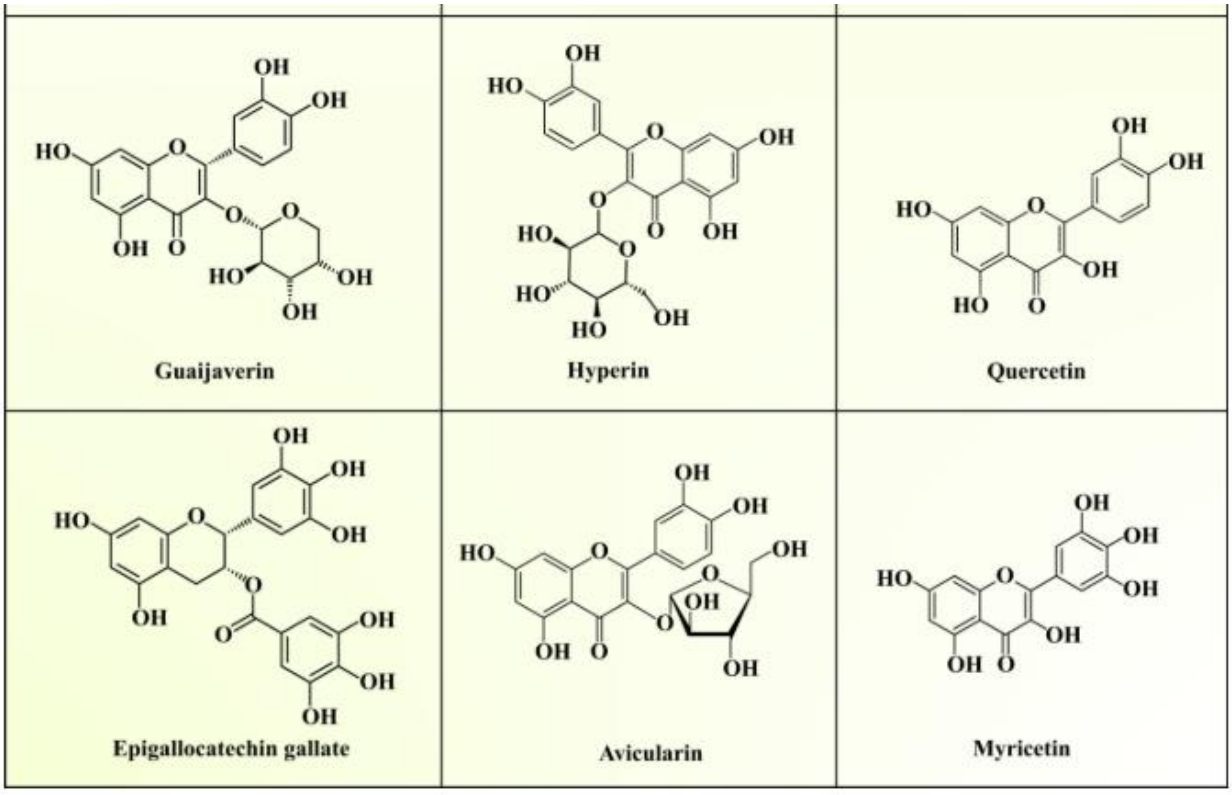


Figure 2.3: Structures of phenolic compounds present in guava leaf extracts.

2.9. Biological Activities of Guava Leaf Extracts

The chemicals included in guava leaf extracts have a wide range of biological effects, including as lowering blood sugar levels and fighting cancer. It was also shown that compared to unsulfated polysaccharide fractions of sulfated GLP, the former exhibited greater biological activities, including antioxidant, antibacterial, and anticancer properties (Kumar et al., 2021). The following sections outline the beneficial bioactivities of GL extract.

2.9.1. Anticancer/Antitumor Activity

Cancer is a complex health disease characterized by increased cell proliferation or reduced cell death (Toyokuni, 2016). Overproduction of reactive oxygen species (ROS) can cause DNA or RNA strand breakage, mutations, base mismatches, chromosomal instability, DNA cross-linking,

degradation of nucleic acids, lipid peroxidation-induced cell membrane damage, and tumor development 47 . Guava leaf extracts, rich in vitamins E, flavonoids, and β-caryophyllene, have been found to have strong inhibitory effects on various cancer cell lines, including colon, prostate, and colorectal cancer. Guava leaf extracts have been found to inhibit angiogenesis, a process that provides tumor cells with essential nutrients and oxygen. Guajadial, a caryophyllene-based meroterpenoid, has been found to have antiproliferative and antiestrogenic effects against human breast cancer cell lines. Guava leaf extracts also inhibited various cancer genes, blocking tumor proliferation, migration, angiogenesis, adhesion, and degradation of the extracellular matrix. Overall, guava leaf extracts have shown potential in treating cancer and preventing the progression of other types of cancer (Huang et al., 2019; Jiang et al., 2020; Lok et al., 2020; Zhu et al., 2019).

2.9.2. Antidiabetic Activity

Diabetes is a serious chronic illness and roughly 10% of the world's population suffers from blood glucose metabolic problems, generally defined by a hyperglycemic state. This state is either characterized by inadequate production of insulin from β-cells of pancreatic islets (type 1 diabetes) or the inability of cells to respond in response to the released insulin (type 2 diabetes) (Mazumdar et al., 2015; Punia & Kumar, 2021). The International Diabetes Federation estimates that 451 million people were affected in 2017, with the prevalence expected to exceed 693 million by 2045 (Cho et al., 2018). Long-term hyperglycemia leads to increased ROS and dyslipidemia, causing serious cellular damage (Hu et al., 2018). Green leafy veg (GLs) have been used as ethnomedicine for diabetes management 15, with flavonoids and polysaccharides showing potential antidiabetic potential (Zhu et al., 2020). Guaijaverin and avicularin flavonoids of GL extract improved pancreatic islet function and hepatocyte morphology in diabetic mice

(Eidenberger et al., 2013; Fujimori & Shibano, 2013). GL polysaccharides (GLPs) have been found to reduce cholesterol, triglycerides, and glycated serum protein content in diabetic rats (Luo et al., 2018; Nair et al., 2013).

2.9.3. Antioxidant Activity

Oxygen is essential for respiration and energy generation, but free radicals can cause various diseases. Phenolic chemicals, such as gallic acid, pyrocatechol, taxifolin, ellagic acid, and ferulic acid, are responsible for the antioxidant effects of guava leaf (GL) extracts. GL extracts contain seven major flavonoids, including quercetin, hesperetin, kaempferol, quercitrin, rutin, catchin, and apigenin (Kumar et al., 2021b). These compounds have been shown to limit the damaging effects of free radicals. GL polysaccharides have been found to protect against oxidative stress, lipid peroxidation, and cell death. GL extracts can also be used as functional food additives (Tran et al., 2020), and silver nanoparticles have shown strong DPPH and ABTS radical cation-scavenging activity (Wang, Xie, et al., 2017).

2.9.4. Antidiarrhea Activity

Diarrhea is a major cause of death in children aged 0-5 years. Pharmaceutical companies are working to find new medications with minimal adverse effects, particularly in underdeveloped countries. Synthetic medications for diarrhea often cause negative effects like constipation, intestinal blockage, and vomiting (Mehra et al., 2013). To address these issues, research is focused on studying bioactive components from therapeutic plants. Green tea leaf extracts (GLs) have been found to have antidiarrheal effects, as demonstrated by studies in rats and rodents. GLs also decreased diarrheal symptoms, including production of interstitial fluid and wetness of fecal droppings. In a study, GL water extracts showed effective antidiarrheal activity in mice. Future research should focus on understanding the molecular mechanisms and long-term toxicity

studies in animal models and human patients to determine the effectiveness and safety of GLs in treating diarrhea (Mazumdar et al., 2015).

2.9.5. Antimicrobial Activity

The emergence of new disease-causing strains and resistance to antibiotics are significant issues, affecting the human body and contributing to worldwide mortality. Food-borne infections are caused by bacteria like *Staphylococcus, Shigella, Salmonella, Bacillus, Escherichia coli, Clostridium, and Pseudomonas* (Ullah et al., 2020). Plant-derived bioactive chemicals, such as guava leaves (GLs), are potential sources of antimicrobials, inhibiting microbial cell wall development, disrupting biofilm formation, repressing DNA replication and transcription, impeding ATP production, suppressing bacterial toxins, and generation of reactive oxygen species (Mickymaray, 2019). GL essential oils exhibit high antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli, Streptococcus faecalis, Staphylococcus aureus, and Bacillus subtilis* (Soliman et al., 2016). Studies have revealed their antioxidant and antiproliferative properties, with phenolic acids, flavonoids, terpenoids, glycosides, and saponins positively correlated with antimicrobial activity. GL extracts also exhibit antioxidant enzyme activity, and their antibacterial activity against Pseudomonas aeruginosa is being investigated (Bose & Chatterjee, 2016; Das & Goswami, 2019; Hirudkar et al., 2020).

2.9.6. Hepatoprotective Properties

Guava leaf extract has been found to improve liver lipid metabolism, enhancing the activity of AMPK and PPARα. It may also improve hepatic insulin resistance. Guava leaf extract can control fatty liver levels, which are linked to diabetes. The liver's role in blood glucose control is closely linked to liver dysfunction, such as hepatic enlargement, steatosis, and fibrosis. Guava leaf extract contains bioactive substances that inhibit glucose absorption, boosting blood glucose

levels (Eidenberger et al., 2013; Fujimori & Shibano, 2013). Elevated flavonoid levels from guava leaf extract can induce insulin resistance and limit glucose and lipid levels in type 2 diabetes mellitus mice (Zhu et al., 2020).

2.9.7. Antiobesity and Lipid-Lowering Activity

Guava leaf extracts (GLs) have been found to have antidiabetic effects and are used to treat diabetes. When administered to diabetic rats, GLs reduced blood glucose levels and improved oral glucose tolerance, preventing weight loss due to poor carbohydrate metabolism. These results were consistent with previous studies (Vinayagam et al., 2018). Guava leaf extracts also contain flavonoids like quercetin, kaempferol, guaijaverin, avicularin, myricetin, hyperin, and apigenin, which have antioxidant properties and health-promoting activities. The presence of glycosides is necessary for these inhibitory effects. In rabbits, ethanol extracts of guava leaves reduced blood triglycerides and low-density lipoprotein levels, while relieving high-density lipoprotein levels. These findings suggest that GLs can help manage hypercholesterolemia, a condition resulting from faulty food choices, heredity, or poor lifestyle (Olaniyan, 2017).

2.9.8. GLs as a Functional Food Ingredient

Recent studies have shown that plant byproducts, such as pomace, seeds, husk/bran/seed coat, peel, and leaves, are essential sources of bioactive chemicals that can be used as functional food components (M. Kumar et al., 2019; M. Kumar, Potkule, et al., 2021; Nishad et al., 2021; Punia & Kumar, 2021). Guava leaf tea (GLT) extract, a functional food and beverage, has been found to have numerous beneficial effects, including antimicrobial, antioxidant, and anti-inflammatory actions (Shaheena et al., 2019). It has also been found to alleviate vascular dysfunction in mice with diet-induced obesity (Díaz-de-Cerio, Rodríguez-Nogales, et al., 2017). GL extract has also been used in the preparation of jelly with pectin, with mass spectrometry analysis confirming its

antioxidant and antimicrobial properties (Sampath et al., 2021b). GL has also been found to be a functional immunostimulant element in fortified meals due to its high amount of antioxidant and phenolic chemicals. Guava leaf tea has been found to be safe in terms of food-drug ointeractions, and it is used by borderline diabetics to reduce blood sugar levels after meals. Additionally, GLs have been found to enhance the quality of eggs by inhibiting the enzyme cyclooxygenase (COX), an inflammatory mediator (Santos et al., 2020). These examples suggest that GL is a valuable source of active chemicals for functional ingredient adds in meals without affecting rheological and sensory qualities.

2.10. EXTRACTION TECHNOLOGIES OF BIOACTIVE INGREDIENTS

The extraction of bioactive compounds from various plant-based food sources is performed using a variety of technologies. These methods are applied in industries like cosmetics, pharmaceuticals, and food, aiming to enhance the processes and operations involved in obtaining these compounds, ensuring their stability and accuracy (Rodríguez-Riera et al. 2014). Regardless of the extraction technique employed, it is crucial to maintain control over environmental factors such as temperature, solvent polarity, and pH of the solution during the extraction process. Various extraction techniques have been utilized for isolating bioactive compounds from tropical fruits (Ghenabzia et al., 2023).

2.10.1. Maceration Method of Extraction

The maceration technique involves extracting bioactive compounds from a solid by immersing it in a liquid solvent. In this solid state, the material contains certain compounds that can dissolve in the solvent to extract the desired bioactive substance. The process can take several days, requiring careful control of external factors like light and temperature to achieve optimal results

(Azwanida, 2015). Additionally, the nature of the solid, the type of solvent, and the chemical characteristics of the compounds being extracted are crucial considerations. Despite its advantages, such as the low cost of setting up the extraction unit, the technique has drawbacks, including the lengthy time required and the large amount of solvent needed, making it an inefficient method (Zhang et al., 2018).

2.10.2. Soxhlet Extraction

Soxhlet extraction is widely regarded as a leading method for extracting analytes from solid materials. Since its invention in 1879, it has been a staple in analytical laboratories. Even today, it serves as a benchmark against which newer extraction methods are evaluated. Extensive research has focused on improving the conventional Soxhlet technique to address its limitations, leading to innovations that reduce both extraction time and the amount of solvent required (Gopalasatheeskumar, 2018). The Soxhlet method thoroughly extracts soluble components using a heated solvent. This technique is efficient, requiring only a small amount of solvent to achieve effective results (Bourdon-García 2017).

2.10.3. Supercritical Fluid Extraction Method

The extraction process is designed to isolate specific compounds from mixtures using solvents such as acetone, methanol, ethanol, and water. In this approach, a supercritical fluid, often CO2, is used alongside co-solvents to improve the separation efficiency. For example, rosemary and guava leaves are placed in a column for extraction. In supercritical fluid extraction, CO2 serves as the primary fluid while ethanol and methanol act as additional solvents (Conde et al., 2016). The system includes components like a pressure regulator, pressure cell, heating and cooling systems, a collection vessel, and a pump. The supercritical fluid is modified, passed through the extraction cell where it penetrates the sample matrix, and dissolves the target compounds. These

dissolved compounds are then carried away from the column at low pressure, with the extracted material collecting at the bottom. The CO2 can be recovered and reused. The process typically operates at pressures of 200-300 bar and temperatures of 45-55°C (Zhang et al., 2018).

2.10.4. Ultrasound Extraction Method

Ultrasonically assisted extraction is a promising method due to its eco-friendliness, rapid processing, and minimal solvent use. This technique utilizes cavitation, where ultrasound waves create bubbles in the solvent. These bubbles collapse, causing the cell walls to rupture and releasing the desired compounds. This process heats the solvent, enhancing the diffusion of the extract and improving the transfer of substances between the solid and liquid phases. There are two main approaches: direct, where probes are inserted into the mixture, and indirect, where the sample is placed in a solvent-filled vessel submerged in an ultrasonic water bath. Research has shown this method's effectiveness in extracting bioactive compounds from tropical fruits, such as Annona muricata by-products, Mangifera indica antioxidant fractions, and *Psidium guajava* leaves (Janarthanan et al., 2020).

2.10.5. Steam Distillation Extraction Method

This method is ideal for extracting natural aromatic substances that are sensitive to heat and may degrade at high temperatures. The steam distillation setup includes a steam generator, a dielectric heater, a condenser, and a collection tank. 25 mg of dried leaf material is processed with 300 ml of water to perform the distillation. The distillation should be carried out for 3-4 hours at the solvent's boiling point. Following the distillation, water and volatile compounds are separated using methyl chlorate (Janarthanan et al., 2020).

2.11. SOXHLET EXTRACTION

Soxhlet extraction is regarded as a continuous solvent extraction method, which utilizes solvents at ambient pressure and boiling temperature, for selective extraction of target compounds from solid compounds (Azmir et al., 2013). Soxhlet extraction is also a common method for the extraction of bioactive compounds, such as lipids, sterols, and fatty acids. Antinelli et al. (2002) introduced an innovative solid/liquid extraction approach that utilizes the Soxhlet method with refluxing diethyl ether as an alternative to the traditional liquid/liquid extraction of fatty acids from RJ using organic solvents. The results showed that the fatty acid content isolated by the two methods ranged from 3.5 to 4.2 %, and no significant difference was observed (Zygler et al., 2012). Notably, the new Soxhlet extraction method requires a less toxic solvent and less time compared with the conventional solvent extraction, suggesting that Soxhlet extraction can be considered an excellent alternative. However, Soxhlet extraction has some inevitable disadvantages such as high temperature, which may increase the possibility of fatty acid degradation in RJ (Saini & Keum, 2018).

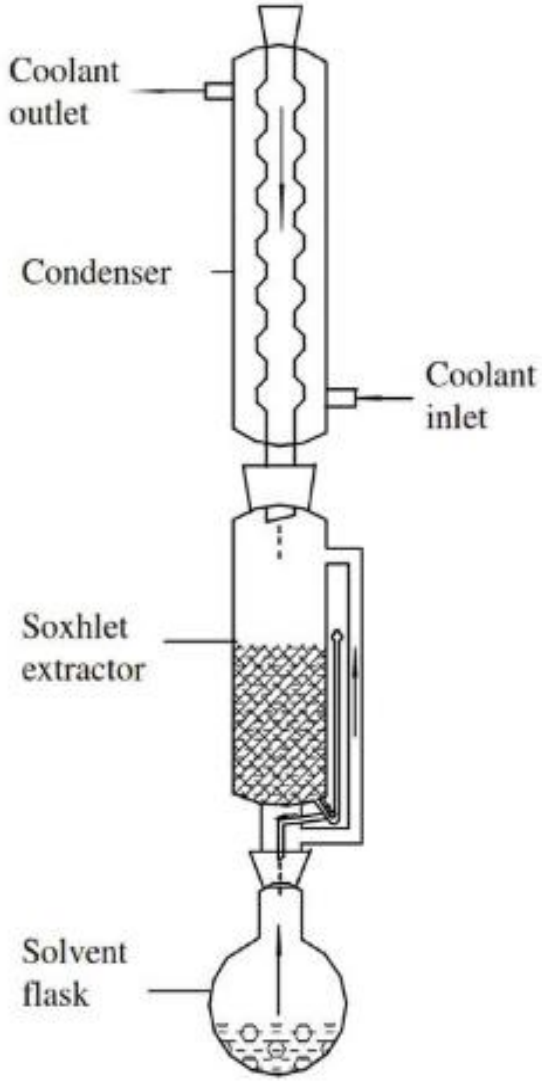


Figure 2.4: Soxhlet extractor (Fotsing et al., 2021)

2.11.1. Soxhlet Extraction of Bioactive Compounds From Herbal Leaves

The process of soxhlet extraction of herbal leaves involves isolating the active medicinal components of a plant using suitable solvents, such as methanol, through established procedures. The Soxhlet apparatus functions on the principle of infusion. Bioactive compounds in the leaves are extracted using this apparatus, which includes a thimble, condenser, siphon tube, bypass tube, reservoir, and a water-cooling system. This method places finely ground herbal leaves in a thimble within a non-woven bag. Methanol is heated in the bottom flask, causing it to vaporize

and pass through the thimble. The vapor then condenses back into the flask after interacting with the plant material. The extraction occurs at a boiling point of 40°C. When the solvent and extract mixture reaches the siphon arm, it settles back into the bottom flask. The extract is then concentrated by removing the solvent using a rotary vacuum evaporator at 30°C, and the final product is stored at 4°C to form a fine powder, which is later dissolved in methanol for further processing (Abubakar & Haque, 2020).

2.11.2. Advantages of Soxhlet Extraction of Bioactive Compounds From Herbal Leaves

Soxhlet extraction is highly efficient for extracting bioactive compounds from herbal leaves. This method allows continuous solvent recycling, ensuring that the plant material is thoroughly extracted. It operates by repeatedly washing the plant material with fresh solvent, which helps maximize the yield of bioactive compounds. The apparatus can run unattended, making it convenient for prolonged extraction processes without the need for constant supervision. Also, Soxhlet extraction is versatile and can be used with a wide range of solvents, making it adaptable to different types of bioactive compounds. It is also a simple and cost-effective method that does not require complex equipment, making it accessible for routine extractions in both research and industrial settings. The ability to perform complete extraction in a relatively short time while minimizing solvent usage is another significant advantage, contributing to both environmental sustainability and cost savings (Kasiramar & K, 2019).

2.11.3. Soxhlet Extraction of Bioactive Compounds From Guava Leaves

Soxhlet extraction is a widely used method for isolating bioactive compounds from guava leaves (*Psidium guajava*). In this process, the guava leaves are first dried and powdered. The powdered leaves are then placed in a Soxhlet extractor, where a solvent, commonly ethanol or methanol, is repeatedly cycled through the sample. The solvent dissolves the desired bioactive compounds as

it passes through the guava leaf material. The process continues until the solvent becomes saturated with the extracted compounds, which are then collected and concentrated after the solvent is evaporated. This method is efficient for extracting a wide range of bioactive compounds, including flavonoids, tannins, and phenolic acids, which are known for their antioxidant, anti-inflammatory, and antimicrobial properties (Sampath Kumar et al., 2021c).

2.12 PREVIOUS RELATED RESEARCH AND DRAWBACKS

Study	**Drawbacks**	**Citation**
Anti-infective efficacy of Psidium guajava L. leaves against certain pathogens	Limited in vivo studies to confirm efficacy in humans.	(Patel et al., 2019)
Susceptibility and Synergistic Effects of Guava Plant Extract and Antibiotics on E. coli	Small sample size; focused only on E. coli, limiting broader applicability.	(Mitra et al., 2024)
Guava Leaf Extract Exhibits Antimicrobial Activity in Extensively Drug-Resistant Bacteria	Variable safety results; more information needed to ensure safe use.	(Gutierrez-Montiel et al., 2025)
Evaluation of a Mouthrinse Containing Guava Leaf Extract	Short-term study; long-term effects and safety not assessed.	(Nayak et al., 2019)
Exploring the Antimicrobial Properties of Guava Leaf Extract Against Selected Food Pathogens	Limited to in vitro studies; in vivo efficacy remains unverified.	(Dzandu et al., 2024)
Influence of the Extraction Method on the Polyphenolic Profile and Antioxidant Activity of Guava Leaves	Focused on polyphenolic compounds; other bioactives not extensively studied.	(Gutierrez et al., 2024)

CHAPTER THREE

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Reagents and Raw Materials Used

The guava leaves were collected from the University of Benin, Benin City, Nigeria, and its neighboring environs.

All chemicals and reagents used were of analytical grade.

The reagents used are listed below;

(i). Distilled water

(ii). Ethanol

(iii). DPPH (2,2-diphenyl-1-picrylhydrazyl) (0.1 mM solution)

(iv). Ascorbic acid (vitamin C)

3.1.2. List of Equipment / Apparatus

(i). Digital scale

(ii). Beakers

(iii). Conical flasks

(iv). Blender

(v). Laboratory pestle

(vi). Laboratory mortal

(vii). Filter paper

(viii). Rotary evaporator

(ix). Oven

(x). freezer

(xi). Petri dish

(xii). Soxhlet apparatus

(xiii). Spectrophotometer

(xiv). Scanning electron microscope (SEM)

(xv). Energy-dispersive X-ray spectroscopy (SEM-EDS)

(xvi). Gas chromatography-mass spectrometry (GC-MS)

3.2. METHODS

3.2.1. Preparation of Powdered Guava Leaves Samples

The guava leaves were dried in the shade to preserve their bioactive compounds. Drying the leaves in the shade helps prevent the degradation of sensitive phytochemicals that might occur if exposed to direct sunlight. This method is chosen to maintain the potency of the leaves' bioactive compounds, such as flavonoids and tannins, which are known for their antioxidant properties. Once sufficiently dried, they were ground into a fine powder using a blender, ensuring a uniform consistency suitable for further extraction processes. The powdered form of the leaves increases the surface area, enhancing the efficiency of subsequent extraction procedures, such as Soxhlet or ultrasound-assisted extraction.

3.2.2. Extraction

The dried leaves were extracted using a Soxhlet apparatus with varying solvent-to-raw material ratios (1:1, 1:4, 1:6), different ethanol concentrations (0%, 5%, 10%), and extraction times (1, 4, 8 hours). The extracts were filtered through filter paper. The filtrate was evaporated to dryness

using a rotary evaporator at 50°C. The resulting crude extract was further dried in an oven at 40°C. The samples were then stored in a freezer at -20°C for further analysis. The yield and total solid content of the guava leaf extract were calculated using the equation below:

$$Yield\ of\ extract\ \left(\%\frac{w}{w}\right) = \frac{g\ of\ solid\ content}{g\ of\ raw\ material}\ \times 100\ \%$$

$$Total\ solid\ content\ \left(\frac{mg}{g}\right) = \frac{g\ of\ dried\ sample\ +\ petri\ dish}{g\ of\ sample\ as\ received}$$

3.2.3. Phytochemical Analysis of Phytochemicals

3.2.3.1. Analysis of Flavonoids

Qualitative: A portion of powdered seed in each case was heated with 10ml of ethyl acetate in a test tube over a steam bath for 3minutes. The mixture was filtered, and 4 ml of the filtrate was shaken with 1ml of dilute ammonia solution. Yellow coloration was observed that indicated the presence of Flavonoids.

Quantitative: A quantity, 0.16 g of the powdered sample was repeatedly extracted with 100 ml of 80% aqueous methanol at room temperature. The solution was shaken for 30minutes and filtrate was transferred into a weighed beaker and evaporated to dryness over a water bath and weighed again. The time for the first extraction was 1 hour, 45minutes for the second extraction and 30 minutes for the third extraction. Flavonoid was determined using the following formula.

Flavonoid (%) = $\frac{W_2 - W_1}{W_3}$ x 100

Where,

W_1 = weight of empty beaker.

W_2 = weight of beaker + sample after drying

W_3 = weight of sample used

3.2.3.2. Analysis of Saponins

Qualitative: 0.2 g portion of the powdered sample was boiled in 20ml of distilled water in a test tube in boiling water bath and filtered. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously to form a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously for the formation of emulsion characteristic of saponins (Obadoni et al., 2001).

Quantitative: A quantity, 0.2 g of the powdered sample was weighed using electric weighing balance into a 250 ml beaker and soaked with 100 ml of 20 % ethanol for three (3) minutes and heated for three (3) hours at 55 0C for proper extraction then filtered. The residue was re-extracted with another 100 ml of 20% ethanol. The two extracts were combined and heated to 40 ml at 90 0C on a water bath. The concentrate was transferred into a 500 ml separating funnel and 20ml of diethylether was added and shaken vigorously, the upper layer was discarded. The purification process was repeated and 60ml of n-batanol was added, the lower layer was discarded while the upper layer was collected. The combined nbutanol extract was washed with 10ml of 5% aqueous NaCI and the lower layer was discarded while the upper layer was collected in a weighed beaker and heated to dryness. The beaker was allowed to cool in a desiccator and reweighed. The saponin content was determined using the following formula.

$$\text{Saponin (\%)} = \frac{W_{2-W_1}}{W_3} \text{ x } 100$$

Where

W1 = weight of empty beaker

W2 = weight of beaker + sample heating

W3 = weight of sample used

3.2.3.3. Analysis of Alkaloids

Qualitative: 0.5g portion of the extract was stirred with 5cm^3 of 1% aqueous HCl on a steam bath. Few drops of picric acid solution were added to 2cm^3 of the extract. The formation of a reddish-brown precipitate was taken as preliminary evidence for the presence of alkaloids.

Quantitative: A quantity, 1.0 g of the powdered sample was weighed using electric weighing balance into a 250 ml beaker and 100ml of 10% acetic acid in ethanol. The mixture was allowed to stand for four hours for proper extraction to take place. The sample was filtered with filter paper and the extract was concentrated on a water bath to one quarter of the original volume. A volume, 20ml of ammonium hydroxide (NH_4OH) was added drop wisely to form precipitate of the alkaloid in the filtrate. The filtrate was weighed with the NH_4OH and filtered. After filtering, the filter paper and the precipitate were dried in an oven at 40^0C and weighed. The alkaloid content was determined using the following formula.

$$\textbf{Alkaloids (\%)} = \frac{W_2 - W_1}{W_3} \; x \; 100$$

Where,

W1 = weight of empty filter paper

W2 = weight of the alkaloid and filter paper,

W3 = weight of sample used

3.2.3.4. Analysis of Tannins

Qualitative: 0.5g portion of the dried powdered sample was boiled in 20ml of distilled water in a test tube and filtered. 0.1% ferric chloride ($FeCl_3$) solution was added to the filtrate. The

appearance of brownish green or a blue-black coloration indicates the presence of tannins in the test samples.

Quantitative: A quantity, 1.0 g of sample powder was weighed into a plastic bottle and 50ml of distilled water was added and shaken for 3 hours in a vibrator. The sample was filtered into a 50ml volumetric flask and made up to mark. A volume, 5ml of the filtrate was dispensed into a test tube and mixed with 2 ml of 0.1M $FeCI_2$ in 0.1NHCI and 0.008 M potassium ferrocyanide, the absorbance was measured at 720nm for 10 minutes. The tannin concentration was determined using the following relation.

$$\text{Tannin (\%)} = \frac{Abs\ x\ DF}{1000a\ X\ Weight\ of\ sample}$$

3.2.3.5. Analysis of Terpenoids (Salkowski test)

Qualitative: Exactly 1.0 g portion of extract was mixed in 2ml of chloroform followed by the careful addition of 3ml of concentrated H_2SO_4. A layer of reddish-brown coloration was formed at the interface thus indicating a positive result for the presence of Terpenoids (Trease and Evans, 1989).

Quantitative: About 1.0 g (Wi) was taken and soaked in 90 ml of ethanol (Indumathi et al., 2014). The extract after filtration was mixed with 10 ml of petroleum ether and again filtrated using separating funnel. The extract was waited for its complete drying and measurement is taken (Wf). The yield (%) of total terpenoids contents was measured by the formula:

$$\textbf{Total Terpenoid} = \frac{\mathbf{W_i - W_f}}{\mathbf{W_i}}\mathbf{x100}$$

Where,

Wi= dried plant extracts,

Wf= extracts after drying

(Activity et al., 2018; Amin Mir et al., 2013; Dahiru et al., 2006; Ezeonu & Ejikeme, 2016; Innocent Izuchukwu Ujah et al., 2021; Krishnananda et al., 2017; Rajiv et al., 2016)

Figure 3.1: Experimental Materials used

3.2.5. Characterization of extracts

The composition of the extracts from the guava leaf was analyzed using FTIR.

3.2.6. Determination of antioxidant activity

The radical scavenging activity of the samples was assessed using a modified version of the DPPH (2,2-diphenyl-1-picrylhydrazyl) analysis method (Nik Mat Daud et al., 2015). Samples were diluted in a series ranging from 1 to 0.0078 mg/mL with 70% ethanol. A 0.5 mL aliquot of each diluted sample was combined with 1 mL of 0.1 mM DPPH solution and mixed well. After a 30-minute incubation period at room temperature in the dark, the absorbance was recorded at 517 nm using a visible spectrophotometer, with 70% ethanol serving as the blank. Ascorbic acid (vitamin C) was used as the positive control under identical assay conditions. The negative control consisted of 2 mL of 0.1 mM DPPH solution and 1 mL of 70% ethanol without any extracts or standards. The antioxidant activity was calculated using the following formula:

$$Antioxidant\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100\ \%$$

$Abs_{control}$ refers to the absorbance measurement of the DPPH solution without any added sample, while Abs_{sample} represents the absorbance measurement of the DPPH solution after the sample has been added.

3.2.7. Experimental Design

During the optimization process, three operating parameters of Soxhlet extraction were optimized using Response Surface Methodology (RSM), a software tool for designing and analyzing experiments. A total of 19 experimental runs were conducted, using three factorial variables to optimize the extraction of guava leaves.

Table 3.1: Processing Parameters of the guava Leaves Extraction Process

Factor	Variable	Unit	Minimum	Mean	Maximum
A	Mass	(g)	1.0000	5.50	10.00
B	Time	(min)	30.00	165.00	300.00
C	Temperature	(°C)	50.00	70.00	90.00

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF THE BIOACTIVE EXTRACT

4.1.2. Functional group of the Bioactive extract

Carbon-containing surface functional groups play an important role in influencing the surface properties and catalytic performance of the calcined scum. These groups can be formed during calcination, where the calcium phosphate scum is heated to high temperatures, leading to the creation of new chemical bonds and surface functionalities. The presence of these functional groups can enhance adsorption capacity, improve chemical reactivity, and contribute to the overall efficiency of the material in catalytic or adsorption applications.

The FTIR spectra obtained for the prepared adsorbent, as revealed in Figure 4.1 and 4.2, provide insights into the molecular vibrations and functional groups present in the flavonoid and terpenoid extract. The analysis of the FTIR spectrum helps in identifying key chemical bonds and interactions, which are critical in understanding the extract's stability, reactivity, and potential applications.

4.1.2.1. FTIR Spectrum of the Terpenoid Extract

These functional groups play a crucial role in determining the extract's antioxidant, antimicrobial, and adsorptive properties, making it a valuable component in various industrial and pharmaceutical applications.

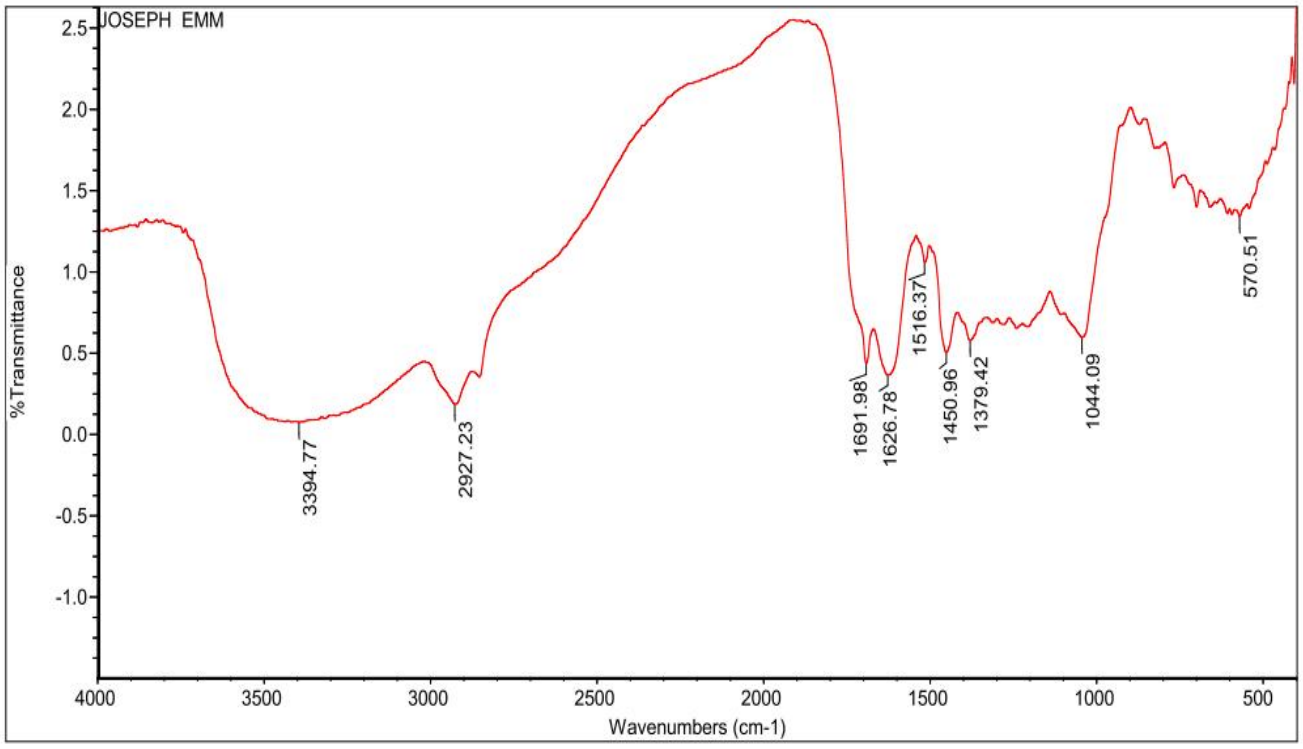


Figure 4.1:FTIR spectrum of flavonoid extract

Table 4.1: ***Summary of Spectrum Peaks of the Flavonoid Extract***

Peak Number	**Position (cm^{-1})**	**Intensity**	**Functional Groups**	**Comment**
1	3394.77	0.0738	O-H (Hydroxyl) Stretching	Indicates the presence of hydrogen bonding and hydroxyl groups, typical in flavonoids.
2	2927.23	0.185	C-H Stretching (Alkanes)	Represents aliphatic C-H stretching, often found in organic compounds.
3	1691.98	0.437	C=O (Carbonyl) Stretching	Commonly associated with flavonoids and ketones.
4	1626.78	0.364	C=C Stretching (Aromatic Rings)	Confirms the presence of aromatic polyphenolic structures.
5	1516.37	1.057	C=C Stretching	Suggests the presence of conjugated

			(Aromatic Rings)	flavonoid structures.
6	1450.96	0.504	CH Bending (Methyl Groups)	Related to alkane bending vibrations.
7	1379.42	0.578	CH Bending (Methyl Groups)	Supports presence of methyl (-CH_3) groups in the compound.
8	1044.09	0.596	C-O Stretching (Ether/Ester)	Indicates possible presence of glycosidic bonds in flavonoids.
9	570.51	1.338	M-O (Metal-Oxygen) Bond	Suggests interaction with metal ions or inorganic components in the extract.

Table 4.3 summarizes the spectrum peaks of the flavonoid extract, highlighting the specific wavenumbers, intensities, and corresponding functional groups. The peak at **3394.77 cm^{-1}** suggests the presence of hydroxyl (-OH) groups, indicating possible hydrogen bonding interactions. Peaks around **2927.23 cm^{-1}** are characteristic of C-H stretching vibrations, typically found in alkanes. The peak at **1691.98 cm^{-1}** corresponds to the stretching of carbonyl (C=O) groups, commonly associated with flavonoids. Peaks at **1626.78 cm^{-1}** and **1516.37 cm^{-1}** indicate C=C stretching vibrations from aromatic rings, confirming the polyphenolic nature of the extract. The peaks at **1450.96 cm^{-1}** and **1379.42 cm^{-1}** suggest the presence of CH bending vibrations, while the strong peak at **1044.09 cm^{-1}** is likely due to C-O stretching in ether or ester groups. Finally, the peak at **570.51 cm^{-1}** could be attributed to metal-oxygen (M-O) bonding, possibly due to interactions with mineral components present in the extract.

4.1.2.2. FTIR Spectrum of the Terpenoid Extract

The FTIR spectrum of the terpenoid extract provides insight into the molecular structure and functional groups present in the sample. By analyzing the characteristic absorption peaks, we can identify key chemical bonds that define the composition of the terpenoid extract.

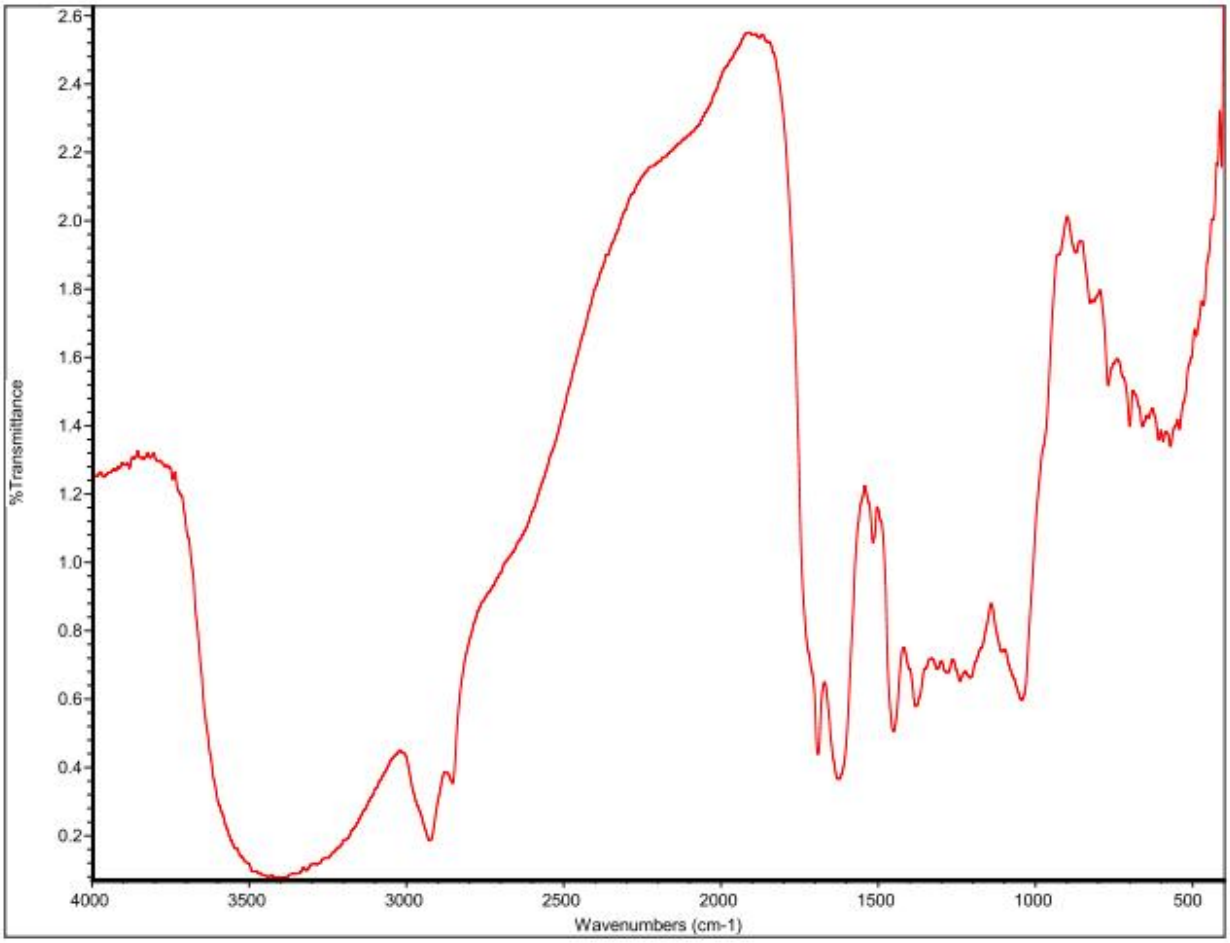


Figure 4.2: FTIR spectrum of the Terpenoid extract

Table 4.2 Summary of Spectrum Peaks of the Terpenoid Extract

Peak Number	Position (cm^{-1})	Functional Groups	Comment
1	3431.33	O-H (Hydroxyl) Stretching	Indicates the presence of hydroxyl groups, suggesting possible alcohol or phenolic content.
2	2760.22	C-H Stretching (Alkanes)	Represents aliphatic C-H stretching, common in terpenoid hydrocarbons.
3	1853.54	C=O (Carbonyl) Stretching	Suggests the presence of ketone or aldehyde functional groups.
4	1643.26	C=C Stretching	Confirms the presence of unsaturated

		(Alkenes)	bonds, characteristic of terpenoid structures.
5	1487.34	C-H Bending (Methyl Groups)	Associated with CH_3 bending vibrations, indicating methyl group presence.
6	1245.98	C-O Stretching (Ether/Ester)	Suggests the presence of ether or ester functional groups, commonly found in oxygenated terpenoids.
7	1226.78	C-O Stretching (Alcohols/Phenols)	Indicates the presence of secondary alcohols or phenolic compounds.
8	1157.90	C-O-C (Epoxide) Stretching	Suggests possible epoxide functional groups, which may influence biological activity.
9	992.72	C=C-H Bending (Alkenes)	Confirms the presence of terminal alkenes, reinforcing the unsaturated nature of the terpenoid extract.

The FTIR analysis reveals key functional groups that define the chemical nature of the terpenoid extract. The presence of hydroxyl (O-H) and carbonyl (C=O) groups suggests oxygenated terpenoids, while the C=C stretching at **1643.26 cm^{-1}** confirms the existence of unsaturated bonds, a common feature in terpenoid compounds. The strong peaks around **1245.98 cm^{-1}** and **1226.78 cm^{-1}** further indicate the presence of ether and alcohol functional groups, which may contribute to the extract's bioactivity.

4.2. QUALITATIVE AND QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS OF PLANT EXTRACT

Table 4.3: Phytochemical Analysis of Guava Leaf Extract

Phytochemical	**Presence (+, -, ++) and colorations'**	**Quantitative Analysis (%)**
Flavonoids	++ (Yellow coloration)	15

Terpenoids	++ (Reddish-brown coloration)	16
Saponins	+ (Stable froth formation)	2
Alkaloids	+ (Reddish-brown precipitate)	1.75
Tannins	+ (Greenish-black coloration)	0.18312

The qualitative analysis confirmed the presence of all tested phytochemicals in guava leaf extract, with flavonoids and terpenoids being significantly present (++), as indicated by their distinct color reactions. The quantitative analysis further supported these findings, showing that flavonoids (15%) and terpenoids (16%) were the most abundant compounds. Saponins (2%), alkaloids (1.75%), and tannins (0.18312%) were also detected, though in lower concentrations. These results highlight the potential of guava leaves as a natural source of bioactive compounds with antioxidant, antimicrobial, and medicinal properties.

4.3. STATISTICAL ANALYZATION AND ANOVA ANALYSIS

From the experimental design, the results obtained are presented in Table 4.4.

Table 4.4: Experimental RSM Result

Run	**Mass of sample (g)**	**Extraction time (min)**	**Temperature (℃)**	**Terpenoid (%)**	**Flavonoid (%)**
1	5.5	165	70	16.923	27.5
2	*10*	*165*	*70*	*11.099*	*18.24*
3	5.5	165	70	16.923	27.5
4	5.5	165	70	16.923	26.23
5	5.5	30	70	10.833	11.53
6	5.5	165	70	16.923	27.5
7	5.5	165	50	7.83	11.21
8	2.82428	84.7285	58.1079	9.273	8.56
9	5.5	300	70	15.4009	17.28
10	8.17572	245.271	58.1079	14.06	23.74

11	8.17572	245.271	81.8921	11.87	15.83
12	8.17572	84.7285	58.1079	10.19	13.41
13	5.5	165	70	12.923	27.5
14	2.82428	84.7285	81.8921	13.392	15.54
15	2.82428	245.271	81.8921	14.33	12.26
16	2.82428	245.271	58.1079	12.61	15.82
17	1	165	70	12.988	11.56
18	5.5	165	90	9.76	11.49
19	8.17572	84.7285	81.8921	9.69	16.58

4.3.1. Quadratic Regression Model and ANOVA for Terpenoid

The experimental data for terpenoid were fitted into a quadratic polynomial model to predict the terpenoid yield based on the independent variables. The final equation in terms of coded factors is:

$$\boldsymbol{Yield\ of\ Terpenoid}\ (\%) = \mathbf{16.09 - 0.5105}\boldsymbol{A} + \mathbf{1.32}\boldsymbol{B} + \mathbf{0.4683}\boldsymbol{C} + \mathbf{0.4683}\boldsymbol{AB} - \mathbf{1.07}\boldsymbol{AC} - \mathbf{0.5111}\boldsymbol{BC} - \mathbf{1.25}\boldsymbol{A^2} - \mathbf{0.8709}\boldsymbol{B^2} - \mathbf{2.40}\boldsymbol{C^2}$$

In this equation, A represents the mass of the sample, B denotes extraction time, and C signifies temperature. Negative coefficients indicate a detrimental effect on terpenoid yield, while positive coefficients suggest a beneficial impact.

ANOVA was conducted to assess the model's significance and the contribution of each term. The analysis revealed that linear terms B (extraction time) and C (temperature) positively influence the terpenoid yield, whereas the quadratic terms A^2, B^2, and C^2 have negative effects, indicating that there are optimal levels for these variables beyond which the yield decreases.

4.3.2. Discussion on the Variable Interactions for terpenoid

The interaction terms AB (mass of sample and extraction time) and AC (mass of sample and temperature) exhibit significant effects on the terpenoid yield. A positive coefficient for AB

suggests that increasing both the mass of the sample and extraction time simultaneously enhances the yield. Conversely, a negative coefficient for AC indicates that increasing the mass of the sample while raising the temperature may reduce the yield.

These interactions underscore the importance of optimizing extraction parameters to achieve maximum terpenoid yield. While higher temperatures can increase extraction efficiency, excessively high temperatures may degrade thermolabile compounds, leading to reduced yields. Similarly, prolonged extraction times can enhance yield up to a point, beyond which degradation or loss of compounds may occur (Nik Mat Daud et al., 2015).

A graphical representation, depicted in Figure 4., illustrates the proximity of these variables, affirming their correlation. The fact that data points in both plots are close to the diagonal line indicates a high level of agreement between expected and observed responses, confirming the model's ability to estimate terpenoid yield effectively.

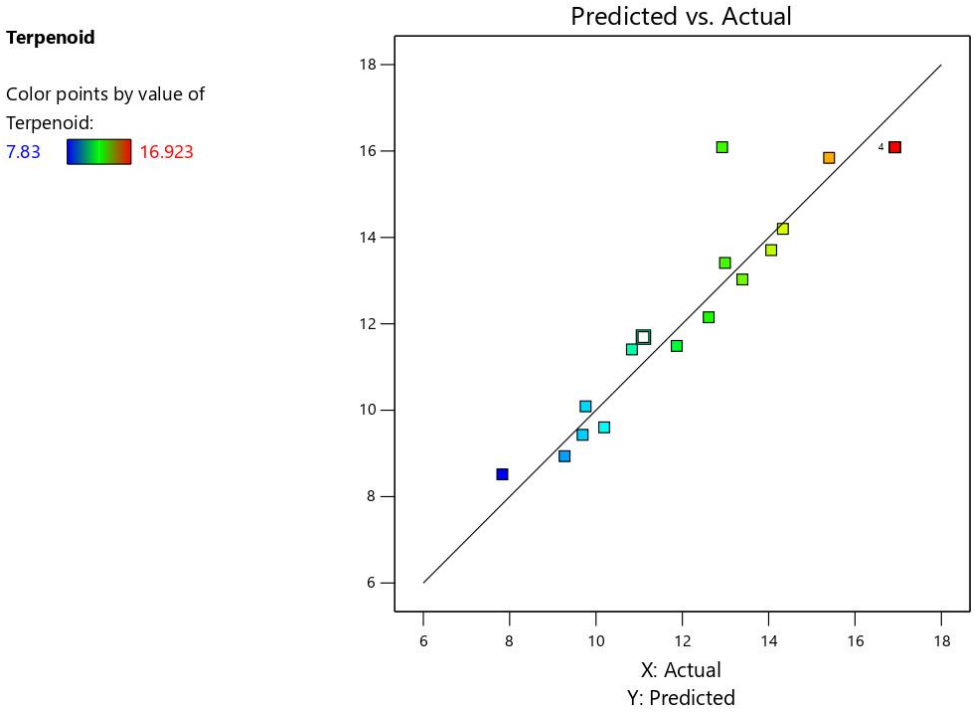


Figure 4.3: Relationship between the predicted and actual value of the yield of terpenoid extract

As shown in Table , the **ANOVA Quadratic Model Analysis of Variance** is a statistical method used to determine the significance of different factors in a model. In the context of a quadratic model analyzing the response variable 'Terpenoid', the ANOVA table provides insights into the contribution of each factor and its interactions. The overall significance of the model is assessed using the F-value and the corresponding p-value.

*Table 4.5: ANOVA Quadratic Model Analysis Of Variance For **Terpenoid***

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	134.00	9	14.89	8.59	0.0019	significant
A-mass	3.56	1	3.56	2.05	0.1856	
B-time	23.74	1	23.74	13.70	0.0049	
C-temp	2.99	1	2.99	1.73	0.2212	
AB	0.3938	1	0.3938	0.2273	0.6449	
AC	9.09	1	9.09	5.25	0.0477	

BC	2.09	1	2.09	1.21	0.3006	
A^2	21.34	1	21.34	12.32	0.0066	
B^2	10.35	1	10.35	5.98	0.0371	
C^2	78.56	1	78.56	45.33	< 0.0001	
Residual	15.60	9	1.73			
Lack of Fit	2.80	5	0.5591	0.1747	0.9585	not significant
Pure Error	12.80	4	3.20			
Cor Total	149.59	18				

An **F-value** of 8.59 with a p-value of 0.0019 indicates that the model is statistically significant, suggesting that the independent variables collectively have a meaningful impact on the response variable. This is further supported by the high F-value, which implies that the variance explained by the model is substantial compared to the unexplained variance.

Examining the individual terms, factors B (time), AC (interaction between mass and temperature), A^2 (mass squared), B^2 (time squared), and C^2 (temperature squared) have **p-values** less than 0.05, indicating their significant contribution to the model. Conversely, factors A (mass), C (temperature), AB (interaction between mass and time), and BC (interaction between time and temperature) have p-values greater than 0.05, suggesting they are not significant contributors. It's important to note that while individual terms may not be significant, they can still be essential for maintaining the model's hierarchy and overall integrity.

The **Lack of Fit F-value** of 0.17 with a p-value of 0.9585 indicates that the lack of fit is not significant relative to the pure error. This suggests that the model fits the data well, as a non-significant lack of fit is desirable

Table 4.6: ANOVA Fit statistical parameters for the quadratic model

R^2	**0.8957**
Adjusted R^2	0.7915
Predicted R^2	0.7247
Adeq Precision	7.9297
Std. Dev.	1.32
Mean	12.84
C.V. %	10.25

The adjusted coefficient of determination (adj R^2) corrects for small discrepancies between experimental and predicted models. A difference of less than 0.3 between adj R^2 and predicted R^2 indicates model significance. Here, adj R^2 is 0.7915 and predicted R^2 is 0.7247, meeting the criterion for significance. The coefficient of variation (CV) reflects data accuracy, with lower values indicating higher accuracy (Dharma et al., 2016). In this study, CV is 10.25%, suggesting reasonable model accuracy. Adequate precision, a measure of signal-to-noise ratio in the analysis of variance, should exceed 4; the model's value of 7.930 indicates sufficient precision (Yahya et al., 2020).

4.3.3. Quadratic Regression Model and ANOVA for Flavonoid

The experimental data for flavonoids were fitted into a quadratic polynomial model to predict the terpenoid yield based on the independent variables. The final equation in terms of coded factors is:

Final Equation in Terms of Coded Factors

$$\boldsymbol{Flavonoid}\ (\%) = \mathbf{26.35} + \mathbf{2.10}A + \mathbf{1.70}B - \mathbf{0.0622}C + \mathbf{0.70}AB - \mathbf{1.02}AC - \mathbf{2.70}BC - \mathbf{3.56}A^2 - \mathbf{3.74}B^2 - \mathbf{4.82}C^2$$

In this equation, A represents the mass of the sample, B denotes extraction time, and C signifies temperature. Negative coefficients indicate a detrimental effect on terpenoid yield, while positive coefficients suggest a beneficial impact.

ANOVA was conducted to assess the model's significance and the contribution of each term. The analysis revealed that linear terms A (mass) and B (extract time) positively influence the flavonoid yield, whereas the quadratic terms A^2, B^2, and C^2 have negative effects, indicating that there are optimal levels for these variables beyond which the yield decreases.

4.3.4. Discussion on the Variable Interactions flavonoids

The interaction terms AB exhibit significant effects on the flavonoid yield. A positive coefficient for AB suggests that increasing both the mass of the sample and extraction time simultaneously enhances the yield. Conversely, a negative coefficient for AC indicates that increasing the mass of the sample while raising the temperature may reduce the yield. The graphical representation in Figure 4., illustrates the proximity of these variables, affirming their correlation. The fact that data points in both plots are close to the diagonal line indicates a high level of agreement between expected and observed responses, confirming the model's ability to estimate terpenoid yield effectively.

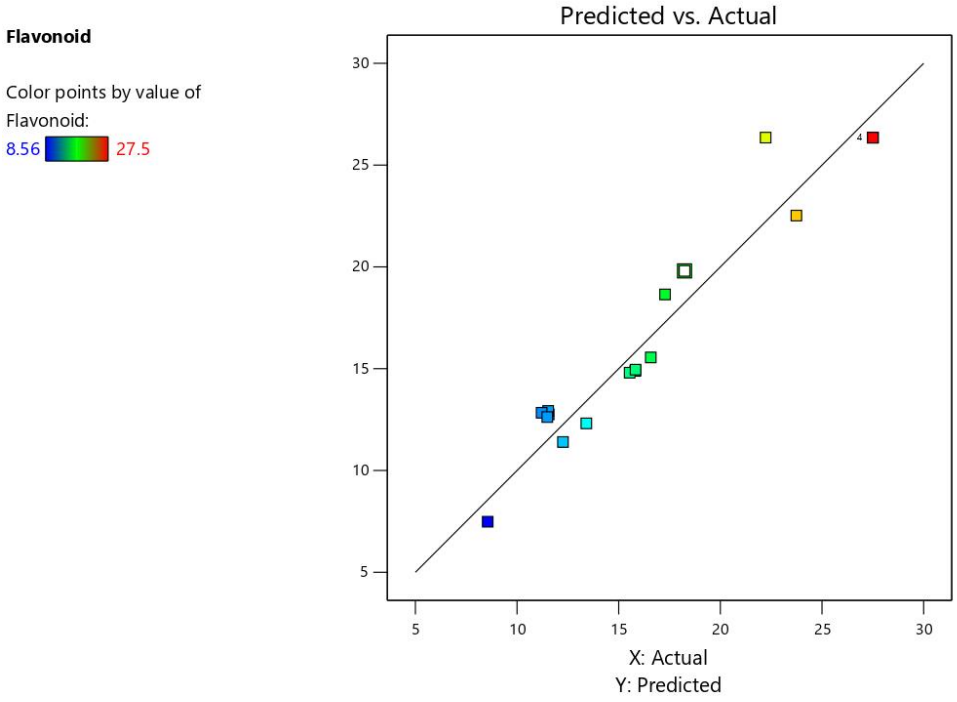


Figure 4.4: Relationship between the predicted and actual value of the yield of flavonoid extract

An **F-value** of 16.80 with a p-value of 0.0001 indicates that the model is statistically significant, suggesting that the independent variables collectively have a meaningful impact on the response variable.

*Table 4.7: ANOVA Quadratic Model Analysis Of Variance For **Flavonoid***

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	700.81	9	77.87	16.80	0.0001	significant
A-mass	59.95	1	59.95	12.94	0.0058	
B-time	39.51	1	39.51	8.53	0.0170	
C-temp	0.0528	1	0.0528	0.0114	0.9173	
AB	3.92	1	3.92	0.8459	0.3817	
AC	8.32	1	8.32	1.80	0.2130	
BC	58.43	1	58.43	12.61	0.0062	
A^2	173.01	1	173.01	37.33	0.0002	
B^2	190.44	1	190.44	41.10	0.0001	

C^2	316.50	1	316.50	68.30	< 0.0001	
Residual	41.71	9	4.63			
Lack of Fit	19.49	5	3.90	0.7017	0.6520	not significant
Pure Error	22.22	4	5.55			
Cor Total	742.52	18				

P-values less than 0.05, indicating their significant contribution to the model. In this case A, B, BC, A^2, B^2, C^2 are significant model terms. have p-values greater than 0.05, suggesting they are not significant contributors.

The **Lack of Fit F-value** of 0.7 with a p-value of 65.20% indicates that the lack of fit is not significant relative to the pure error. This suggests that the model fits the data well, as a non-significant lack of fit is desirable

Table 4.8: ANOVA Fit statistical parameters for the quadratic model

R^2	**0.9438**
Adjusted R^2	0.8877
Predicted R^2	0.7551
Adeq Precision	12.0810
Std. Dev.	2.15
Mean	17.65
C.V. %	12.20

The adjusted coefficient of determination (adj R^2) corrects for small discrepancies between experimental and predicted models. A difference of less than 0.3 between adj R^2 and predicted R^2 indicates model significance. Here, the adjusted R^2 is 0.8877, and the predicted R^2 is 0.7551, meeting the criterion for significance. The coefficient of variation (CV) reflects data accuracy, with lower values indicating higher accuracy. CV is 12.20%, suggesting reasonable model accuracy. Adequate precision, a measure of signal-to-noise ratio in the analysis of variance, should exceed 4; the model's value of 12.081 indicates an adequate signal.

4.4. THE EFFECT OF PROCESSI NG PARAMETERS ON THE YIELD OF EXTRACT (FLAVONOID AND TERPENOID) YIELD

4.4.1. The Impact Of Time And Mass On The Yield of the Extract

Figures 4.5 and 4.6 showed that time and mass significantly impacted the yield of terpenoids and flavonoids. In both cases, at a constant temperature, an increase in time and mass initially led to a slight increase in terpenoid yield. This is attributed to bioactive compounds' enhanced diffusion and dissolution as more solvent interacts with the plant matrix over time. However, at masses above 55 g and extraction times beyond 165 minutes, the yield progressively diminishes. This drop is explained by the solvent reaching its saturation point, limiting further solubilization of the compounds. Also, prolonged extraction times led to the degradation of thermolabile compounds due to extended exposure to heat and solvent interaction and excessive mass loading resulted in poor solvent penetration and hindered effective mass transfer, reducing the overall efficiency of the extraction process.

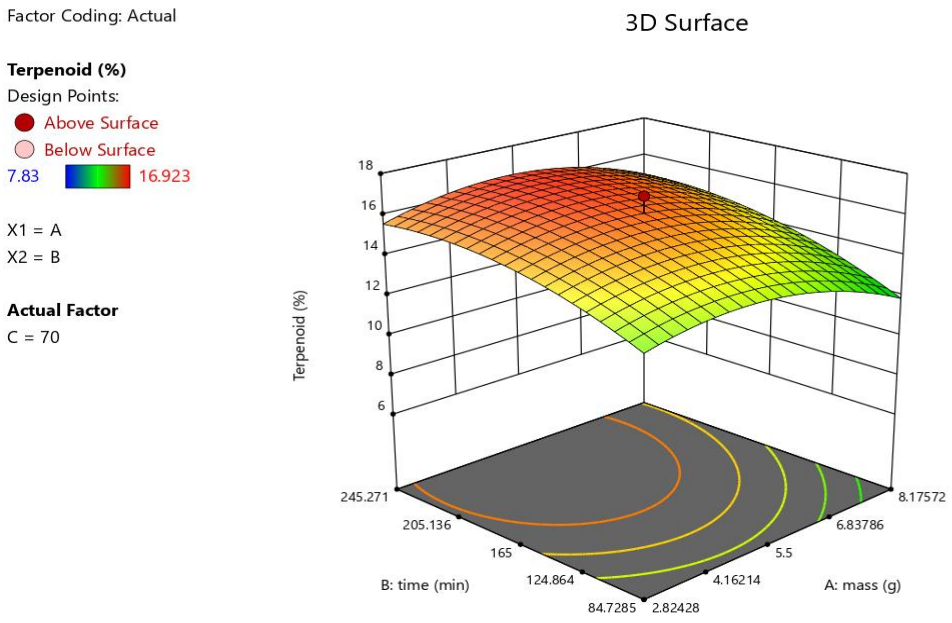


Figure 4.5: 3D Response surface plot showing the combined effect of time of extraction to mass of sample on the yield of Terpenoid extract

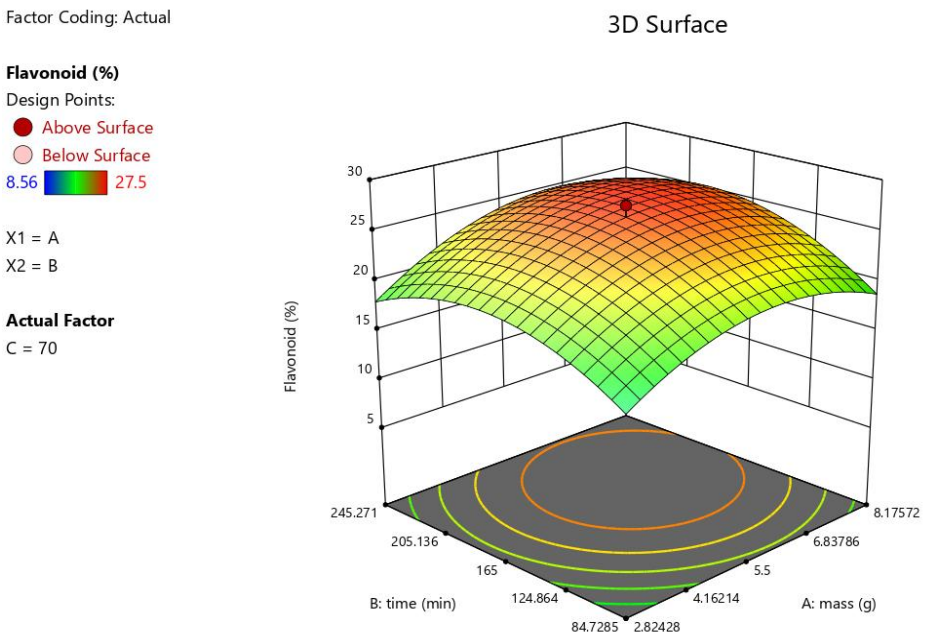


Figure 4.6: 3D Response surface plot showing the combined effect of time of extraction to mass of sample on the yield of Flavonoid extract

4.4.2. The Impact Of Temperature And Mass On The Yield of the Extract

From Figure 4. and 4., at constant time, the temperature and mass significantly impacted the yield of both terpenoids and flavonoids. The yield of terpenoids experienced a slight increase, shortly followed by a rapid decrease. This is due to the fact that moderate temperature and mass enhance the solubilization and diffusion of bioactive compounds, but excessive temperature and mass can lead to degradation of thermolabile compounds and reduced extraction efficiency. Elevated temperatures may also cause volatilization or structural breakdown of certain terpenoids, leading to a decline in yield.

For flavonoid extract (Figure 4.), it is observed, as expected, that the yield began to decrease at a mean temperature of 70°C and mass of 165 g. This is explained by the degradation of flavonoids at high temperatures, as they are heat-sensitive compounds. Also, excessive mass can result in reduced solvent availability per unit mass, leading to poor extraction efficiency due to insufficient solvent penetration and limited mass transfer.

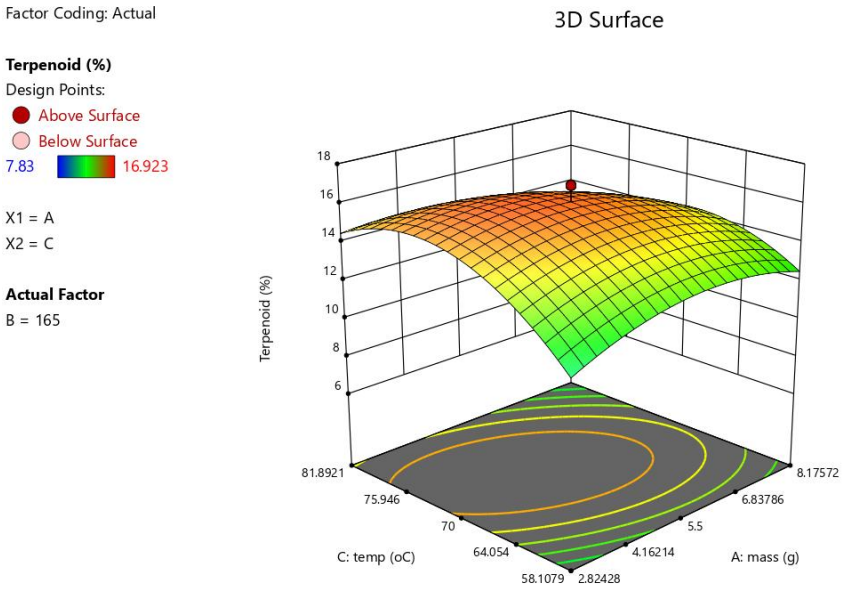


Figure 4.7: 3D Response surface plot showing the combined effect of temperature of extraction and mass of sample on the yield of Terpenoid extract

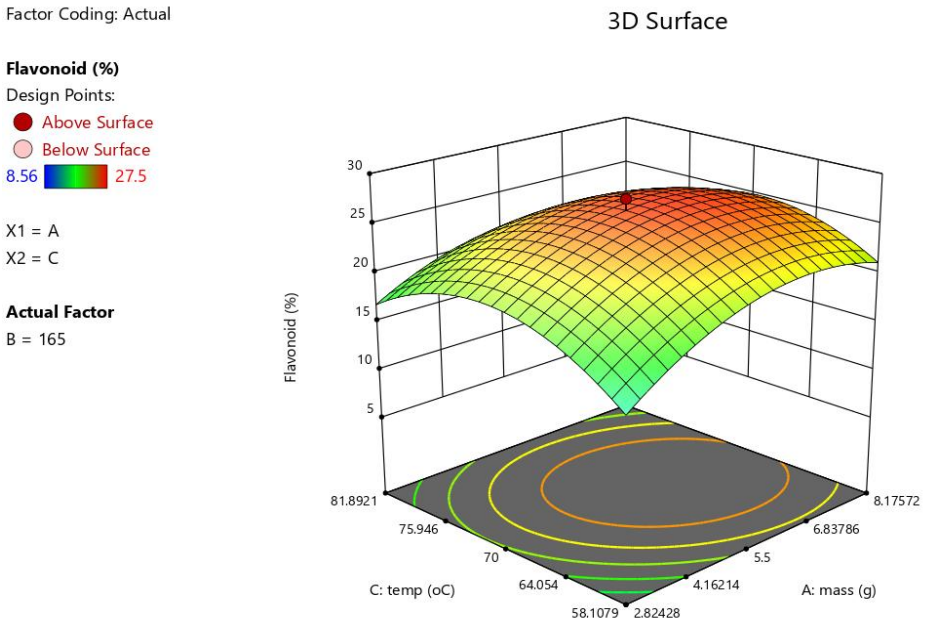


Figure 4.8: 3D Response surface plot showing the combined effect of temperature of extraction and mass of sample on the yield of Flavonoid extract

4.4.3. The Impact Of Temperature And Time On The Yield of the Extract

An increase in temperature favors the extract yield with increasing time and constant mass for both terpenoids and flavonoids, as depicted in Figure 4. and 4. This is attributed to the enhanced solubility and diffusion of bioactive compounds at elevated temperatures, which facilitates improved mass transfer from the plant matrix into the solvent. However, beyond the mean temperature of 70°C, there is a decline in yield for both cases.

This investigation reveals that above 70°C, the bonds of the extract break, leading to thermal degradation of bioactive compounds. Also, the degraded components may undergo polymerization or oxidation, reducing their solubility in the solvent. Furthermore, excessive heat can cause some volatile compounds to evaporate, while others may begin to absorb back into the plant matrix or precipitate out of the solution, thereby reducing the overall yield of the extracted terpenoids and flavonoids.

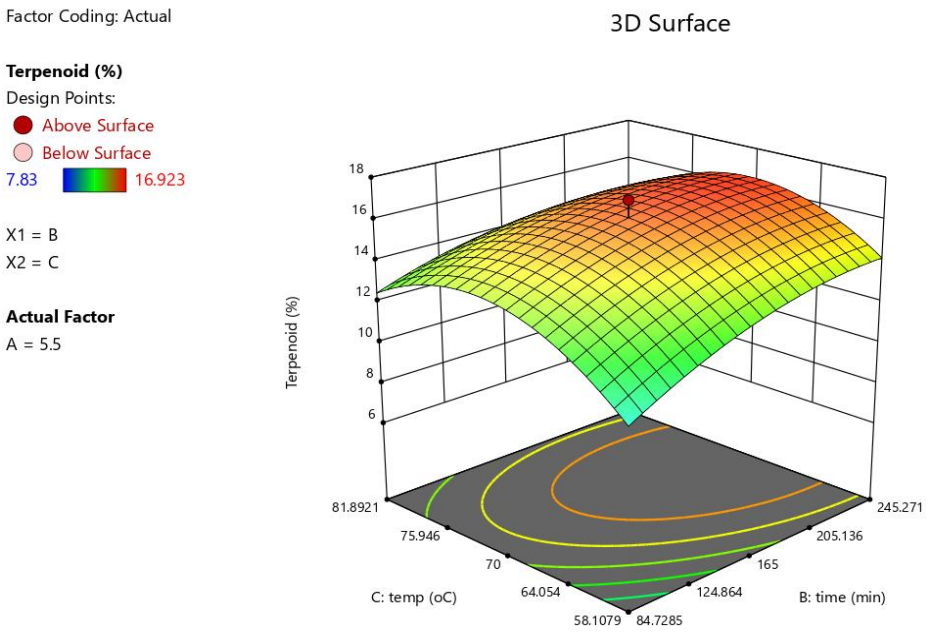


Figure 4.9: 3D Response surface plot showing the combined effect of temperature of extraction and time on the yield of Terpenoid extract

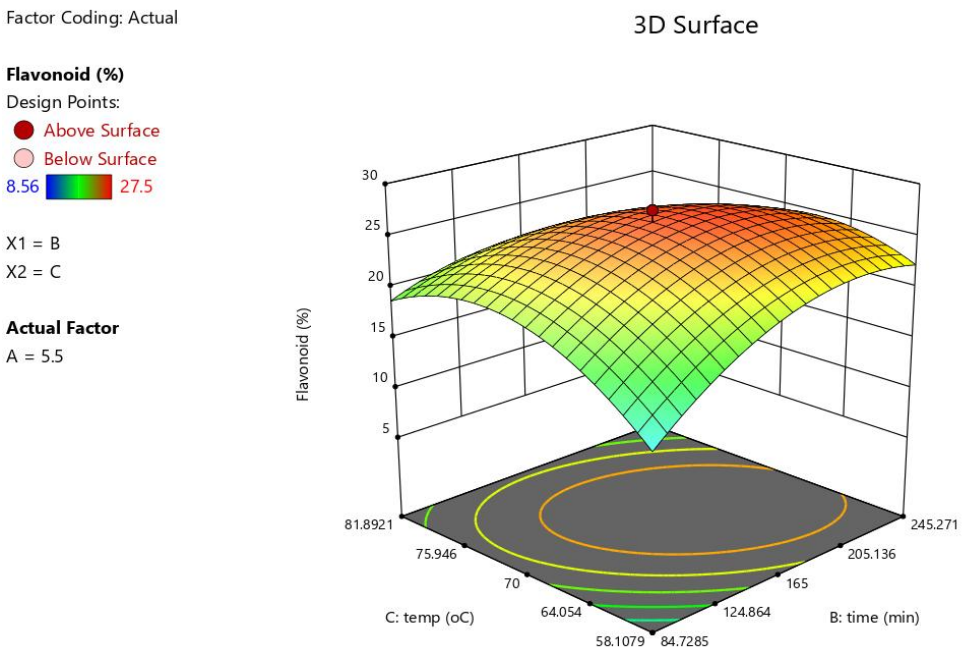


Figure 4.10: 3D Response surface plot showing the combined effect of temperature of extraction and time on the yield of Terpenoid extract

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1. Conclusion

In conclusion, this study successfully optimized the extraction of bioactive compounds from guava leaves using Soxhlet extraction and Response Surface Methodology (RSM), demonstrating a rigorous and systematic approach. The key extraction parameters—solvent mass, temperature, and extraction time—were precisely controlled to maximize yield.

Comprehensive characterization confirmed the presence of flavonoids, terpenoids, saponins, alkaloids, and tannins. The regression models for terpenoid and flavonoid yields exhibited strong predictive accuracy, with **R^2 = 0.7915 for terpenoids** and **R^2 = 0.8957 for flavonoids**. ANOVA analysis further validated the model significance (**F-value = 0.17, p = 0.9585** for terpenoids and **F-value = 8.59, p = 0.0019** for flavonoids), reinforcing the reliability of the optimization process. These findings establish guava leaves as a **highly promising source of bioactive compounds** with significant pharmaceutical and therapeutic potential.

5.2. Recommendations

Based on the findings of this study, the following recommendations are proposed:

(i). Further research should be conducted to fine-tune extraction conditions, such as solvent type, temperature, and time, to maximize the yield of bioactive compounds.

(ii). Other extraction techniques such as ultrasound-assisted extraction should be investigated, to demonstrate high efficiency in obtaining antioxidant compounds from guava leaves

(iii). The effectiveness of different solvents, including hydroethanolic mixtures, should be evaluated to enhance the extraction of phenolic compounds, which are closely linked to antioxidant activity

(iv). Advanced analytical methods like UPLC-MS to identify and quantify specific bioactive compounds in guava leaves, should be utilized as this aids in the development of targeted therapeutic applications.

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