ANTIOXIDANT ACTIVITIES & POLYPHENOL CONTENTS IN *Rhododendron arboreum* KOMBUCHA FLAVORED WITH STRAWBERRY, YELLOW BERRY AND BLACK GRAPES

Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Science

In

Biotechnology

By

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Under the supervision of

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To

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in this report entitled **"Antioxidant activities & polyphenol contents in *Rhododendron arboreum* kombucha flavored with strawberry, yellow himalayan berry and black grapes"** in complete fulfillment of the requirements for the award of the degree of **Master of Science** in **Biotechnology** submitted in the Department of Biotechnology & Bioinformatics**,** Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from August 2022 to December 2022 under the supervision of **Dr. Anil Kant** , Associate Professor, Department of Biotechnology and Bioinformatics.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to certify that the project work **"Antioxidant activities & polyphenol contents in *Rhododendron arboreum* kombucha flavored with strawberry, yellow himalayan berry & black grapes"** by **Sumangala Kejriwal** during their end semester in fulfillment for the award of degree of Masters of Science in Biotechnology from Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially to any other University or Institute for the award of any degree or appreciation.

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Sumangala Kejriwal

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LIST OF ABBREVIATION

SCOBY- Symbiotic Culture Of Bacteria and Yeast

TPC- Total Phenolic Content

TFC- Total Flavonoid Content

DPPH- 2,2 Diphenyl-1-picrylhydrazyl

UDP- Uridine Diphosphate

FCR- Folin – Ciocalteu Reagent

MHA- Mueller Hinton Agar

MHB- Mueller Hinton Broth

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ABSTRACT

The aim of this dissertation was to examine the potential antioxidant, polyphenolic and antimicrobial properties of *Rhododendron arboreum* kombucha with added flavors of strawberry, yellow berry, and black grapes. The kombucha was produced using a standard fermentation process with black tea, *Rhododendron arboreum* and honey as substrates in the 1st fermentation, and the fruits as flavors were added during the second fermentation. The antioxidant activity was deliberated using the DPPH assay, while the total flavonoid and phenol contents were determined using colorimetric methods. The results indicated that all three flavored kombucha samples had higher antioxidant, total phenol, and total flavonoid contents than the control sample without added flavors. Black grapes had the maximum of total phenol content and antioxidant activity, while yellow berry had the highest total flavonoid content. The antimicrobial activity was tested against four microorganisms using the agar well diffusion method. All three flavored kombucha samples exhibited significant inhibition against the tested microorganisms, with black grapes flavor showing the highest activity. The findings suggest that *Rhododendron arboreum* kombucha with added flavors would be a potential source of natural antioxidants and antimicrobial agents.

Keywords: *Rhododendron arboreum*, Kombucha, Black Grapes, Strawberry Yellow Himalayan Berry, antioxidant activity, antimicrobial activity, total flavonoid and phenolic content.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

Kombucha is an increasingly popular fermented beverage known for its potential health benefits. It is prepared by fermenting tea and sugar with the help of a symbiotic culture of bacteria and yeast (SCOBY). The SCOBY is a mixture of different bacterial species including *Gluconacetobacter, Acetobacter,* and *Lactobacillus*, as well as yeast species like *Saccharomyces, Brettanomyces*, and *Candida*. These microorganisms convert the tea and sugar into a slightly tangy and effervescent beverage [5].

Traditionally, black tea has been used as the base for brewing kombucha. However, it is also possible to use green tea or other herbal teas as a starting point. Kombucha can be further enhanced by adding various fruits and spices to create different flavors. This versatility in flavoring allows for a wide range of taste experiences and makes kombucha appealing to different preferences.

It is worth noting that while kombucha is generally considered safe for consumption, individuals with certain health conditions or compromised immune systems should exercise caution or consult with a healthcare professional before incorporating it into their diet. Additionally, homemade kombucha should be prepared with proper hygiene and care to prevent the growth of harmful bacteria.

The Himalayan rhododendron (*Rhododendron arboreum*) is a plant species indigenous to the Himalayas and other Asian mountainous areas. It has a long history of use in Ayurvedic medicine due to its potential health benefits. Previous research has demonstrated that the plant possesses antioxidant and antimicrobial properties. Furthermore, it contains a variety of bioactive compounds (for example phenolic compounds and flavonoids), which are recognized for their beneficial effects on health [14].

The purpose of this dissertation is to assess the antioxidant activity, total phenol content (TPC), total flavonoid content (TFC), and antimicrobial activity of *Rhododendron arboreum* kombucha infused with three different fruits: strawberry, yellow himalayan berry, and black grapes. The study also seeks to understand how fruit flavoring affects the antioxidant and antimicrobial properties of kombucha, and to explore the potential of *R. arboreum* kombucha as a functional beverage. The results of this investigation could provide valuable insights into the health-promoting qualities of kombucha and the potential utilization of *R. arboreum* as a functional ingredient in food and beverage applications.

The objective of my dissertation work are as follow:

1. Preparation of kombucha using dried flowers of *Rhododendron arboreum*.
2. Flavouring of kombucha using strawberry, yellow himalayan berry, and black grapes fruits.
3. Quantitative analysis of TPC, TFC and antioxidant property of kombucha.
4. Assessment of antimicrobial activity, pH and sugar of kombucha.

CHAPTER 2: REVIEW OF LITERATURE

2. KOMBUCHA

Kombucha, a fermented beverage originating from Asia, is traditionally made using black tea (*Camellia sinensis*) sweetened with sugar. However, alternative tea varieties like green tea and oolong tea can also be used. The typical method involves boiling 1 litre of water and adding 5-10 grams of tea leaves and 50-70 grams of sugar. After 5 minutes of stirring, the tea leaves are removed, and the mixture is cooled to around 20°C. Then, 3% of the SCOBY and 0.2% of previously brewed kombucha tea are added to start the fermentation process. The container is covered with a breathable muslin cloth to allow for oxygen exchange. Fermentation occurs over approximately 21-30 days within the ideal temperature range of 18-26°C [23].

The Symbiotic Colony of Bacteria and Yeasts (SCOBY), which is a cellulose film, primarily comprises of acetic acid bacteria (such as *Gluconacetobacter xylinus, Acetobacter malorum, Komagataeibacter xylinus*), yeast (such as *Candida parapsilosis, Zygosaccharomyces bailii, Saccharomyces cerevisiae*), and lactic acid bacteria (such as *Lactobacillus bulgaricus, Lactobacillus plantarum*). These microorganisms are essential for the fermentation process. Yeasts that thrive in high-sugar environments ferment the tea's sugar, converting it into ethanol, which is then metabolized by bacteria into acetic acid. The SCOBY is composed of a floating biofilm and a tangy liquid phase. Both the liquid and biofilm contain compounds like acetic acid, gluconic acid, and ethanol, contributing to the distinctive characteristics of kombucha. The cellulose film of the SCOBY possesses a notable ability to absorb water.

Following the fermentation process, kombucha undergoes chemical changes and contains a diverse range of components. These include minerals like iron (Fe), nickel (Ni), calcium (Ca), magnesium (Mg), manganese (Mn), copper (Cu), and zinc (Zn). It also contains organic food acids, carbon dioxide, polyphenols, dietary fibre, water-soluble vitamins such as vitamin C, and amino acids (like lysine), sugars, and antibiotic compounds, various forms of vitamin B, hydrolytic enzymes, and ethanol [13].

Kombucha has gained popularity as a functional beverage due to its numerous benefits, including antioxidant activity and anti-inflammatory potential (Fig: 2.1). It has been found to have positive effects on reducing blood pressure, inhibiting cancer growth, and improving the immune system, as well as supporting liver and gastrointestinal function.

These wide-ranging advantages have prompted further research into the practical applications of kombucha and its impact on the microbiome and overall health.

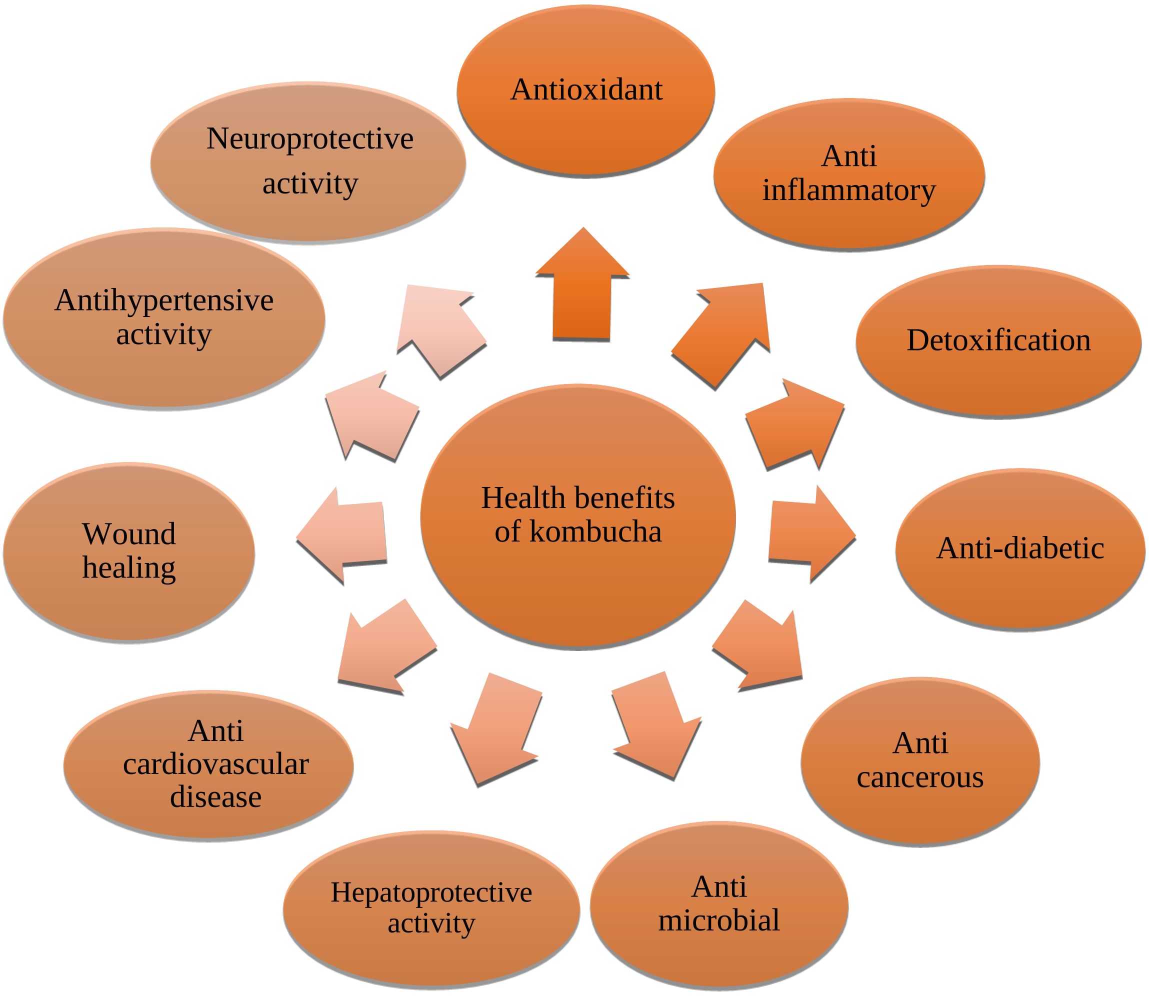

Fig 2.1: Health benefits of Kombucha

The liquid broth of kombucha contains minor and major components such as carboxylic acid, gluconic acid, acetic acid, glucuronic acid, ethanol, and phenolic compounds including epicatechin gallate, catechin, epigallocatechin gallate, epicatechin, and epigallocatechin. Additionally, enzymes and vitamin B are also present. The composition of the liquid broth contributes significantly to its immune-modulating properties and is believed to play a role in potential therapeutic effects, including anti-carcinogenic, antioxidant, and liver detoxification properties.

The popularity and accessibility of kombucha tea have been enhanced by its availability in various forms and flavors, as well as the support of numerous scientific studies confirming its properties [13]. According to a study by Zhou et al. (2022) [3], fermenting kombucha with tea residue could be a more effective approach for producing kombucha with higher levels of antioxidants, specifically polyphenols.

2.1 Chemical components of Kombucha:

Kombucha is a complex beverage that contains a diverse array of chemical constituents, encompassing organic acids, vitamins, biogenic amines, purines, proteins, enzymes, pigments, amino acids, lipids, microorganisms, sugars, carbon dioxide, minerals (such as copper, zinc, manganese, iron, nickel, lead, cadmium, cobalt, and chromium), anions (bromide, iodide, fluoride, chloride, phosphate, nitrate, and sulfate), polyphenols, DSL (D-saccharic acid-1,4-lactone), and various metabolic by-products.

Kombucha beverages have been found to contain a wide range of chemical compounds. These include various organic acids for example gluconic acid, acetic acid, and glucuronic acid (GlcUA). Additional acids such as citric acid, L-lactic acid, malic acid, tartaric acid, succinic acid, malonic acid, oxalic acid, usnic acid, and pyruvic acid may also be present. Sugars such as glucose, fructose, and sucrose are commonly found, along with water-soluble vitamins including B1, B2, B6, B12, and C. Acetic acid bacteria, lactic acid bacteria and metabolic products of yeasts and bacteria have also been identified as components of kombucha beverages [20].

2.2. Types of Tea:

While black tea is traditionally used for brewing kombucha, alternative varieties of tea can be utilized to impart distinct flavors and potential health benefits. There is a range of common tea types that can be employed in the production of kombucha. Some common types of tea that can be used in kombucha are as follows:

2.2.1. Black Tea:

Black tea is obtained from the leaves of the *Camellia sinensis* plant and is produced through a specific series of steps. The leaves undergo a withering process, followed by rolling or crushing, and are then left to oxidize for a period of time. This oxidation process alters the color of the leaves from green to a deep brown or black shade, resulting in the characteristic flavor and aroma of black tea. It is a widely consumed beverage globally, especially in Western countries, and is commonly enjoyed with the addition of milk and sugar [6].

Black tea offers notable advantages due to its elevated caffeine content, which can enhance alertness and focus. Additionally, it contains theanine, an amino acid that aids in relaxation and stress reduction. Rich in antioxidants, black tea provides protection against various ailments, including cancer, cardiovascular disorders, and diabetes. Numerous studies have demonstrated its efficacy in lowering blood pressure and diminishing the risk of stroke

2.2.2. Green Tea:

Green tea is obtained from the leaves of the *C. sinensis* plant, and it stands out for not undergoing an oxidation process. Instead, the leaves are steamed or pan-fried, which helps retain their inherent flavor and aroma. Green tea holds great popularity as a beverage in Asian nations, especially in Japan and China, where it is frequently enjoyed as an integral part of traditional tea ceremonies.

Green tea is highly valued for its substantial antioxidant content, specifically catechins, which contribute to its potential in safeguarding against a range of illnesses such as cancer, cardiovascular disease, and diabetes. Another notable component found in green tea is theanine, known for its ability to induce relaxation and alleviate stress. Numerous studies have indicated that green tea can effectively reduce blood pressure, lower LDL cholesterol levels, and enhance cognitive function [6].

2.2.3. White Tea:

The youngest leaves and buds of the *C. sinensis* plant, specifically picked before they reach full maturity. These tender parts are withered and dried without undergoing oxidation or rolling, ensuring the preservation of their inherent taste and fragrance. White tea is considered relatively uncommon and comes with a higher price tag, predominantly enjoyed in China.

White tea is renowned for its notable concentration of antioxidants, particularly polyphenols, which contribute to its potential in combating diseases such as cancer, cardiovascular disorders, and diabetes. Additionally, white tea boasts a significant content of catechins, which can enhance the immune system and support skin health. Various studies have indicated that white tea can effectively reduce blood pressure and mitigate the likelihood of stroke [6].

2.2.4. Oolong Tea:

Oolong tea is created through a unique process involving partially oxidized leaves from the *Camellia sinensis* plant. These leaves undergo withering, rolling, and a shorter duration of oxidation compared to black tea. The resulting oolong tea can exhibit a diverse range of flavors and aromas, influenced by the degree of oxidation and the specific processing methods employed. Widely enjoyed in China and Taiwan, oolong tea is frequently served alongside meals in these regions.

Oolong tea offers notable advantages due to its abundant antioxidants, primarily theaflavins and catechins, which contribute to its potential in safeguarding against diverse ailments such as cancer, cardiovascular disease, and diabetes. Furthermore, oolong tea's caffeine content can enhance alertness and concentration. Several studies have indicated that oolong tea can aid in weight loss efforts and diminish the likelihood of heart disease.

2.2.5. Herbal Tea:

Herbal teas are prepared by utilizing dried flowers, leaves, roots, or fruits from a variety of plants, including chamomile, peppermint, ginger, and hibiscus. They are flavored as caffeine-free alternatives to traditional teas. Herbal teas are renowned for their wide-ranging health advantages, including enhanced digestion, anxiety reduction, improved sleep quality, and alleviation of inflammation [6].

2.3. SCOBY:

The symbiotic culture of bacteria and yeast (SCOBY), which is commonly known as kombucha culture, is a biofilm that arises from the cooperative interaction between yeasts and acetic bacteria. When cultivated in a static environment, it takes on a fungal carpet-like appearance, earning it the nickname "fungus tea." This biofilm develops on sweetened tea that has been cooled, forming a film composed of cellulose [4].

SCOBY consists of a diverse array of microorganisms, including bacteria from the *Lactobacillus* species, *Acetobacteraceae* family (such as *Acetobacter aceti, Komagataeibacter kombuchae, Gluconobacter oxydans, K. rhaeticus, A. estunensis, A.*

pasteurianus, and *K. xylinus*), and osmophilic yeasts (including *Brettanomyces/Dekkera, Candida, Starmerella* species, *Pichia* species, *Saccharomyces, Schizosaccharomyces, Torulopsis,* and *Zygosaccharomyces*). During the fermentation process of kombucha, these microorganisms utilize sucrose as their primary carbon source, with nitrogen provided by the tea extract. In the aerobic condition, the SCOBY produces carbon dioxide, organic acids, and a floating biofilm composed of cellulose. Over time, this cellulose-based biofilm gradually thickens, often forming multiple layers resembling pancakes. The primary function of this cellulose pellicle is to safeguard the SCOBY from external contaminants [28].

Within kombucha, the yeast community transforms sucrose into glucose and fructose, with glucose being further converted into ethanol. Simultaneously, acetic acid bacteria utilize glucose through the action of glucose oxidase, resulting in the production of organic acids like glucuronic, acetic, and gluconic acid. Additionally, fructose is converted into acetic acid. This dynamic interaction between yeast and bacteria leads to the increase of ethanol and acetic acid in the kombucha, serving as antimicrobial agents that impede the growth of harmful microorganisms [9].

During the oxidative fermentation process, the acetic acid bacteria undergo specific pathways to generate the cellulose biofilm. The biosynthesis of cellulose involves a series of enzymatic reactions facilitated by essential enzymes. Glucose undergoes transformations into glucose-6-phosphate and then into glucose-1-phosphate. The conversion of glucose-1-phosphate into UDP glucose is catalysed by uridine diphosphate (UDP)-glucose pyrophosphorylase, which serves as a precursor for cellulose synthesis. Glucosyltransferase enzymes utilize UDP glucose as a substrate, and among them, cellulose synthase is a key player. Cellulose synthase incorporates additional units of UDP glucose, rapidly forming a polymer chain referred to as β-1/4 glucan chain at an impressive rate of 200,000 residues per second (D. Laavanya, 2021). A benefit of this cellulose production process is that the bacteria grow under controlled conditions and can utilize various substrates such as glucose, ethanol, and sucrose [9].

2.3.1. Application of SCOBY:

Recent research has uncovered the vast potential of SCOBY in various fields, including biotechnology, food science, and medicine. Apart from its role in kombucha production, SCOBY has been harnessed for the fermentation of diverse foods and beverages, expanding its applications in the culinary domain.

SCOBY also exhibits promise in the realm of bioplastics manufacturing. Bioplastics are a category of plastics derived from sustainable sources like plants or microorganisms. Notably, a research study has demonstrated the utilization of SCOBY to produce a specific form of bioplastic known as bacterial cellulose. This bacterial cellulose possesses excellent biodegradability and holds significant potential for applications in medicine and biotechnology [4].

SCOBY has demonstrated promising applications in wastewater treatment as well. Research indicates that SCOBY can effectively aid in the removal of heavy metals, such as lead, copper, and zinc, from wastewater. The bacteria and enzymes present in SCOBY have the ability to bind to these heavy metals, facilitating their extraction from the water. This process enhances the safety of the water for both human consumption and environmental discharge.

Furthermore, SCOBY shows promise in the realm of food packaging, particularly as a biodegradable packaging material and a bio-composite film used in meat packaging. The dried biofilm sheets derived from SCOBY can be utilized as protective covers for preserving vegetables over extended periods without compromising their nutritional value. Additionally, an active bio-composite film, incorporating chitosan and kombucha, can be employed to package minced meat, thereby extending its shelf life [4].

The extensive biomedical applications and significance of SCOBY are evident in its frequent use for skin repair treatments, particularly in cases involving burns, wounds, and ulcers. SCOBY membranes accelerate the process of proliferative phase of wound healing and prevent infections. Moreover, SCOBY biocomposites show promise in regulating cell adhesion, a crucial characteristic for scaffolds and grafts. Additionally, the utilization of ultra-thin films derived from bacterial cellulose (BC) holds potential in the development of diagnostic sensors, thanks to their ability to immobilize various antigens [9].

2.4. Additives:

2.4.1. *R. arboreum*

Rhododendron, a genus of vascular plants, is widely distributed across the Northern Hemisphere, encompassing a significant number of species. There are approximately 1200 estimated species of *Rhododendron* worldwide, with China boasting the highest number of species at 571, including 409 endemic species. In India, there are around 80 species, 10 subspecies, and 14 varieties. The state of Arunachal Pradesh is known to host the highest number of species, with 67 species, followed by Sikkim with 36 species. Darjeeling district in West Bengal has 19 recognized species, while Nagaland, Manipur, Mizoram, and Meghalaya each have 7, 5, 2, and 2 species, respectively. Uttaranchal has 6 species, and Himachal Pradesh and Jammu & Kashmir have 4 species each. Tamil Nadu and Kerala have one subspecies each (Fig 2.2). Among the various species, *R. arboreum* stands out as an impressive and majestic species. It exhibits considerable variability in terms of flower color, height, hardiness, and leaf characteristics. The name of the species "*arboreum*" reflects its tree-like appearance [2].

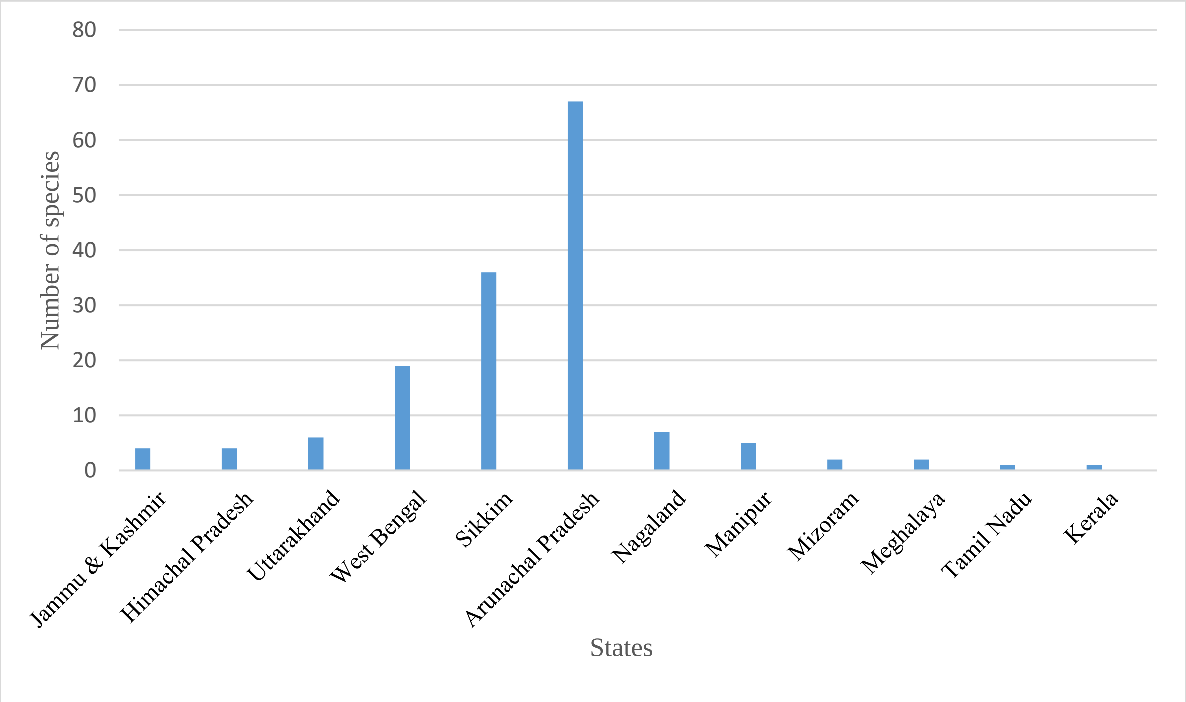

Fig 2.2: Distribution of *Rhododendron* in India [2].

Table 2.1: Taxonomic classification of *R. arboreum* [26].

Kingdom	Plantae
Phylum	Magnolliophyta
Class	Angiospermae
Order	Ericales
Family	Ericaceae
Genus	*Rhododendron*
Species	*Rhododendron arboreum*

2.4.1.1. Chemical components of *R. arboreum*.

Rhododendron is rich in a variety of minerals, including iron, zinc, copper, cobalt, nickel, lead, manganese, cadmium, molybdenum, sodium, chromium, and arsenic. These minerals play essential roles in maintaining vital physiological processes necessary for life. Important minerals such as manganese, selenium, zinc, iron, copper, and molybdenum act as critical cofactors in specific enzymes and are involved in various biochemical pathways. Sodium is crucial for maintaining the osmotic balance between cells and the surrounding interstitial fluid.

Furthermore, *Rhododendron* contains abundant secondary metabolites like alkaloids, saponins, flavonoids, tannins, steroids, glycosides, and phlobatannins. These secondary metabolites serve important functions in the plant's survival and also contribute significantly to human health [27]**.**

2.4.1.2. Medicinal properties:

In Far-West Nepal, *Rhododendron* has been traditionally employed as a medicinal remedy due to its wide range of therapeutic properties and minimal side effects. The twigs and leaves of *Rhododendron* are a source of phenolic acids, which have been found to exhibit anti-inflammatory, anti-HIV and anti-nociceptive activities. Additionally, the leaves and flowers of the plant are utilized in the cure of various conditions including illness, headache, diabetes, and rheumatism [27].

R. arboreum had traditional use as both an analgesic and sedative agent. It had been found to possess anti-inflammatory properties by effectively preventing the production of pro-inflammatory cytokines and mediators [18].

2.4.2. Yellow Himalayan raspberry (*Rubus ellipticus*)

The yellow himalayan berry is a plant that thrives in tropical climates and wet forests. While its origin can be traced back to moderate Himalayas region, it is inhabitant to Southeast Asia, Currently, it could be found in various areas of the Himalayas, spanning from Pakistan to Nepal and Southern China. Additionally, it is also found in countries such as Myanmar, Burma, Sri Lanka, Philippines, and Thailand [11].

2.4.2.1 Chemical components of *Rubus ellipticus:*

The yellow Himalayan berry fruit contains several phenolic compounds, antioxidants, flavonoids, phytochemicals, and other bioactive compounds. These constituents significantly contribute to the fruit's nutritional value, distinguishing it from other fruits. Some of the major chemical constituents of *Rubus ellipticus* are:

2.4.2.1.1. Ellipticine:

Ellipticine, an alkaloid found in the roots and stems of *Rubus ellipticus*, exhibits anticancer properties and has been employed in the management of different cancer forms [17].

2.4.2.1.2. Quercetin:

Quercetin, a flavonoid found in the leaves and stems of *Rubus ellipticus*, possesses antioxidant and anti-inflammatory properties. It has demonstrated potential therapeutic uses in the treatment of diverse ailments [17].

2.4.2.1.3. Ursolic acid:

Ursolic acid, a triterpenoid found in the leaves and stems of *Rubus ellipticus*, exhibits anti-inflammatory, anti-tumor, and anti-diabetic properties, as reported by studies[17].

2.4.2.1.4. Cyanidin-3-rutinoside:

Cyanidin-3-rutinoside, an anthocyanin found in the fruit of *Rubus ellipticus*, possesses antioxidant and anti-inflammatory properties. It has been identified as having potential therapeutic applications in the treatment of various diseases [19].

2.4.2.2. Medicinal properties:

Yellow Himalayan raspberry, scientifically known as *Rubus ellipticus*, has a rich history in traditional medicine, where it has been utilized for various therapeutic purposes. This plant contains a diverse range of compounds that have demonstrated medicinal properties. For instance, extracts derived from *Rubus ellipticus* leaves have shown promising anti-inflammatory effects by preventing the production of pro-inflammatory cytokines and mediators. Additionally, these extracts exhibit potential in managing diabetes by reducing blood glucose levels and enhancing insulin sensitivity. The presence of ellagic acid and other polyphenols in the plant may contribute to its anti-cancer properties. *Rubus ellipticus* has also exhibited hepatoprotective activity, safeguarding the liver against damage induced by toxic substances. Furthermore, the plant exhibits antimicrobial activity against several strains of bacteria and fungi.

2.4.3. STRAWBERRY (*Fragaria × ananassa*)

Strawberries are grown in various Asian countries, including China, Japan, South Korea, and India. In Japan, strawberries hold significant value as a crop and are carefully cultivated in greenhouses to shield them from the harsh winter weather. Japanese strawberries are known for their smaller size and enhanced sweetness compared to strawberries grown elsewhere. They are frequently incorporated into desserts, particularly strawberry shortcake. In China, strawberries are predominantly grown in the southern regions, and their harvest season typically spans from April to June [19].

2.4.3.1 Chemical component:

Strawberries contain various bioactive compounds including phenolic acid, flavonoids, anthocyanins, vitamin C, etc.

Phenolic acid: Strawberries contain a class of compounds called phenolic acids, which possess antioxidant and anti-inflammatory properties. These compounds are recognized for their potential in combating cancer. Among the phenolic acids found in strawberries, ellagic acid is the most prevalent.

Flavonoids: Strawberries contain a variety of flavonoids, which are compounds known for their antioxidant properties. Among the flavonoids present in strawberries are quercetin, kaempferol, and catechin.

Anthocyanins: Anthocyanins are pigments which is the reason for the red color of strawberries, possess antioxidant properties and are linked to various health benefits. These include promoting heart health and reducing the risk of chronic diseases like cancer and diabetes.

Vitamin C: Strawberries are rich in vitamin C, an antioxidant that plays a crucial role in defending the body from oxidative stress [7].

2.4.3.2. Medicinal properties:

For centuries, strawberries have been utilized in traditional medicine. The presence of flavonoids and phenolic acids in strawberries contributes to their anti-inflammatory properties. Ellagic acid, specifically found in strawberries, has demonstrated potential anti-cancer effects. Additionally, the flavonoids and anthocyanins in strawberries have been linked to improved heart health. In overweight individuals, strawberry consumption has been associated with enhanced lipid profiles and reduced oxidative stress. Moreover, the flavonoids and anthocyanins in strawberries show promise in the realm of diabetes management by reducing blood glucose levels in individuals with type 2 diabetes. Lastly, the antioxidants present in strawberries, such as vitamin C, possess anti-aging properties [7].

2.4.4. Black Grapes (*Vitis vinifera*):

Black grapes, scientifically known as *Vitis vinifera*, are a globally prevalent grape variety. They are renowned for their deep hue and flavorful taste, making them highly sought-after for both the production and enjoyment of wine.

2.4.4.1 Chemical composition:

Black grapes, scientifically known as *Vitis vinifera*, are abundant in bioactive compounds such as polyphenols, anthocyanins, flavonoids, and resveratrol. These compounds possess an extensive range of health-promoting properties, including anti-cancer, anti-inflammatory, antioxidant, and heart-protective effects.

Black grapes are particularly rich in polyphenols, which are a diverse group of bioactive compounds. These compounds can be classified into different subclasses, including flavonoids, phenolic acids, and stilbenes. Among these subclasses, flavonoids are the most abundant in black grapes and encompass compounds like quercetin, kaempferol, and myricetin. Phenolic acids, such as gallic acid and caffeic acid, are also present in black grapes, while resveratrol, a type of stilbene, has gained significant recognition for its positive effects on health [8].

Anthocyanins, which are vital bioactive compounds, can also be found in black grapes. These compounds contribute to the deep color of black grapes and are primarily concentrated in the skin rather than the flesh. The predominant anthocyanins in black grapes include delphinidin, petunidin, and malvidin.

In addition to the aforementioned bioactive compounds, black grapes also contain a range of other essential components. These include various vitamins like vitamin C, vitamin K, and vitamin B6. Additionally, minerals like potassium, calcium, and magnesium can be found in black grapes. Furthermore, black grapes serve as a source of carbohydrates, specifically glucose and fructose, which are the primary providers of energy [8].

2.4.4.2. Medicinal properties:

Black grapes are rich in polyphenols, well-known for their anti-inflammatory and antioxidant properties. Antioxidants play a crucial role in counteracting the harmful effects of free radicals, which can harm cells and promote the development of chronic illnesses like cancer, cardiovascular disease, and neurodegenerative disorders. Similarly, anti-inflammatory compounds aid in reducing inflammation within the body, which is linked to various chronic diseases [12].

The polyphenols in black grapes have beneficial effects on cardiovascular health by reducing blood pressure, improving blood circulation, and preventing blood clot formation. They also possess anti-cancer properties, preventing the growth and spread of cancer cells. Additionally, the presence of resveratrol, a stilbene compound, in black grapes contributes to their cardioprotective effects, including reducing the risk of atherosclerosis and improving the function of blood vessels. The high antioxidant content in black grapes provides anti-aging benefits by protecting against oxidative stress, which can damage cells and accelerate the aging process [12].

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials and chemicals required

Black tea, honey, drinking water, previous batch raw kombucha, dried *R. arboreum* flower, 5L airtight glass jars, muslin cloth, black grapes, strawberry, yellow Himalayan raspberry, gallic acid, methanol, distilled water, Folin-Ciocalteu reagent (FCR), 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, sodium bicarbonate, sodium nitrate, aluminium chloride, Mueller Hinton agar (MHA), sodium hydroxide, Mueller Hinton broth (MHB), petri plates.

3.2 Methodology

3.2.1 Collection of plant sample

The flower of *R. arboreum* was collected from the forest of Palampur, Dharamshala, Himachal Pradesh. The Plants were authenticated in Dr. YS Parmar University of Horticulture and Forestry by preparing a Herbarium sheet. The herbarium number of plant samples was 13775.

3.3 Collection of fruit sample

Yellow Himalayan Berry were collected from the jungle of Waknaghat, Solan. Strawberry and Black Grapes were collected from local market of Solan, Himachal Pradesh

3.4. Preparation of *R. arboreum* Kombucha (1st Fermentation)

Tea was prepared by an infusion of 30g of black tea in 4 L of boiling water. Honey was infused at a concentration of 50ml/L and 6.25g/L, 8.75g/L, 11.25g/L concentrations of *R. arboreum* dried flower (as composition shown in table 3.1) in boiling water for 10 mins. After 10 min, tea was allowed to cool down to room temperature and after cooling each sample was transferred into glass jars and inoculated with 100ml/L of raw kombucha. The jar mouths were covered with muslin cloth and the fermentation was carried out in a dark room for 30 days at 22°C [6]. Quantitative analysis was analysed on 7, 14, 21, 30 days of fermentation.

Table 3.1: Composition of kombucha

No. of Tests	Composition
1.	30g tea+25g flower+200 ml honey+400 ml raw kombucha+ 4L water
2.	30g tea+35g flower+200 ml honey+400 ml raw kombucha+ 4L water
3.	30g tea+45g flower+200 ml honey+400 ml raw kombucha+ 4L water

3.5. Flavoring with fruits (2nd Fermentation)

Flavors were added to increase its antioxidant property, phenolic and flavonoid content. After 30 days of fermentation, SCOBY form was removed and residues were filtered. A Kombucha sample containing 35g flower was divided in 3 parts of 500ml each, to this added 100ml puree of strawberry, black grapes, yellow himalayan berry, respectively. Each jars were airtight packed and kept for fermentation in a dark room at 22℃ for 4 days. After fermentation, each sample was filtered using whatman filter papers and stored in refrigerator.

3.6. General procedure for total phenolic content (TPC)

In accordance with Gorinstein et al, (2004), the TPC was determined using the Folin-Ciocalteu method, a spectrophotometric assay. 100µl of sample was mixed with 100µl FCR, then the solution was allowed to stand for 5 min in dark before addition of 1 ml Na_2CO_3. Then the solution was kept for 30min in dark at room temperature, the OD was taken at 760 nm. The gallic acid was used as a standard and expressed as µgGAE/ml of sample. *Rhododendron* kombucha samples were analysed on 7, 14, 21, 30 day of fermentation and each sample of fruit flavoured kombucha on 4 day of fermentation.

3.7. General procedure for total flavonoid content (TFC)

The total flavonoid content (TFC) of the sample was measured using the aluminium chloride method, which is a common spectrophotometric assay. Quercetin, a flavonoid compound, was used as a standard to compare the flavonoid content of the sample. 1g of

Quercetin was dissolved in 100 ml methanol to get concentration of 1% Quercetin. Different dilutions of gallic acid were prepared for standard graphs. 150µl of sodium nitrate was added, then incubated for 5 minutes. Then 150µl of Al_2Cl_3 was added, then incubated for 5 minutes, then 200µl of sodium hydroxide was added. Record the absorbance at 510nm. Flavonoid content measured in terms of µgQE/ml of sample.

3.8. General procedure for antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Fresh 0.002% DPPH was prepared in methanol. Different dilutions of gallic acid were prepared for standard graphs. Samples were added to 3ml of DPPH and allowed for 30 min incubation at room temperature in a dark room. Record the absorbance at 517nm and calculate % inhibition by the given formula,

$$\% \text{ inhibition} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

3.9. Antimicrobial activity

To assess the antimicrobial activity of samples, the agar well diffusion method was used following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). Microbial culture (*B. subtilis, S. typhi, E. coli, and S. aureus*) with a concentration of 106 colony-forming units (cfu)/ml for each strain was spread onto Mueller Hinton agar plates using a sterile swab. Wells with a diameter of 8 mm were then created in the agar medium and loaded with 100 µl of the kombucha samples. After two hours at room temperature for the sample to diffuse, the petri plates were kept at 37°C for 24 hours. The diameter of the growth inhibition zones were evaluated in millimetres, and controls were included with distilled water as negative controls and ampicillin as positive controls.

3.10. Determination of sugar content

Total sugar content was measured using a refractometer from °Brix scale.

3.11. Determination of pH

pH of the sample was measured using an electronic pH meter.

CHAPTER 4: RESULT AND DISCUSSION

4.1 Result of preparation of kombucha

Firstly, *R. arboreum* flower were collected and sun dried for 2-3 days. The colour of flower changes from red colour to pinkish red (Fig 4.1). Then tea was prepared with an infusion of black tea, honey and three different concentrations of *R. arboreum* dried flower, 6.25g/L, 8.75g/L, 11.25g/L concentration respectively in 4L of water each and boiled for 10-15 mins (Fig 4.2). Each tea sample was cooled to room temperature and transferred to clean and sterile glass jars. Raw kombucha at a concentration of 100ml/L was added to each sample to initiate the fermentation process. As the fermentation process progressed, a cellulose-based biofilm known as SCOBY developed on the surface of tea. Over time, this biofilm gradually thickens, often forming multiple layers resembling pancakes (Fig 4.3, Fig 4.4 and Fig 4.5). Each jars were stored in dark room at room temperature for 30 days. After 30th day, each sample were filtered and SCOBY was separated. The filtered kombucha obtained was reddish brown in colour (Fig 4.6) and stored in refrigerator.

Fig 4.1: *R. arboreum* dried flower

Fig 4.2: Preparation of tea

Fig 4.3: SCOBY formation after 10 days of fermentation

Fig 4.4: SCOBY formation after 20 days of fermentation

Fig 4.5: SCOBY formation after 30 days of fermentation

Fig 4.6: Filtered kombucha after 1st fermentation

4.2 Result of Flavored kombucha (2nd fermentation)

After 1st fermentation of kombucha, total phenolic content, total flavonoid content, antioxidant property, anti-microbial activity, pH and sugar were analysed for comparative study between different concentrations of *R. arboreum* in kombucha. The results showed that kombucha containing 8.75g/L concentration (T2) of *R. arboreum* dried flower had the maximum antioxidant activity, total flavonoid content and zone of inhibition against *S. aureus, B. subtilis, S. typhi, E. coli.* Kombucha containing 8.75g/L concentration (T2) of *R. arboreum* dried flower was further flavored with strawberry, yellow himalayan berry and black grapes to enhance its properties. Kombucha was divided into 3 parts 500ml each. Then the fruits were crushed to turn in puree (as shown in Fig 4.7). Then the puree we transferred into kombucha (as shown in Fig 4.8) and stored in dark room at room temperature for 4 days. After 4 days of fermentation flavored kombucha was tested for its total phenolic content, total flavonoid content, antioxidant property, anti-microbial activity, pH and sugar.

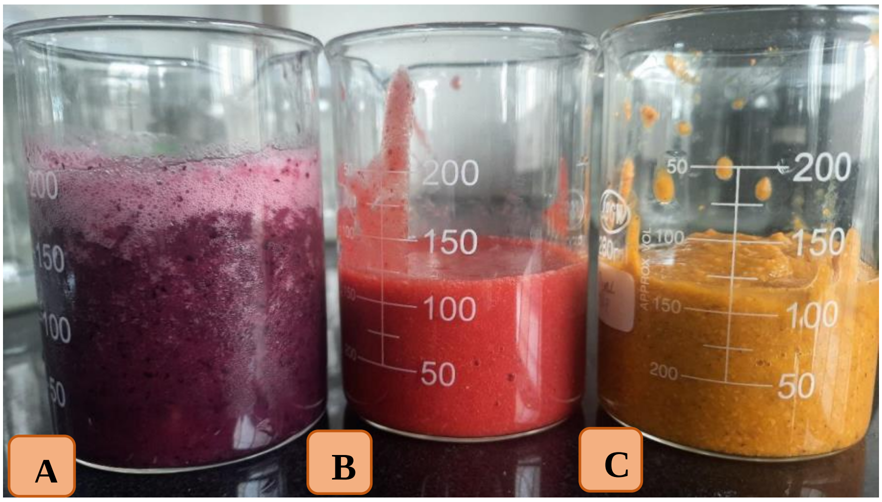

Fig 4.7: Pulp of [A] Black Grapes, [B] Strawberry, [C] Yellow Himalayan Berry.

Fig 4.8: [A] Black Grapes infused *Rhododendron* kombucha, [B] Yellow Himalayan Berry infused *Rhododendron* kombucha and [C] Strawberry infused *Rhododendron* kombucha.

4.3. Result of total phenolic content

The table 4.1 showed the result of phenolic tests conducted on different days of fermentation process of kombucha containing different concentration of dried flower *R. arboreum*. The concentration of phenolic content was measured in µgGAE/ml (micrograms of gallic acid equivalent per millilitre) for each samples. The concentration increases as the fermentation process progressed to the 30 days. On 7th day of fermentation, concentration observed to be highest in T3 (11.25 g/ml concentration of flower) with 0.05737 µgGAE/ml, followed by T2 (8.75g/ml concentration of flower) with 0.04318 µgGAE/ml and lowest in T1 (6.25g/ml concentration of flower) with 0.0379 µgGAE/ml. As the fermentation progressed to the 30th day, the concentration increased to its highest level with 0.3905 µgGAE/ml in T1, 0.4247 µgGAE/ml in T2 and 0.5787 µgGAE/ml in T3.

The table 4.2 showed the result of phenolic content in strawberry, yellow himalayan berry and black grapes flavored kombucha. The black grapes exhibited the highest phenolic concentration of 1.1862 µgGAE/ml, followed by yellow himalayan berry had the concentration of 0.8193 µgGAE/ml and the strawberry kombucha had the lowest concentration of 0.5608 µgGAE/ml.

Overall, the result showed that the phenolic content of *R. arboreum* kombucha increases over time. Kombucha containing 11.25g /L concentration (T3) of *R. arboreum* dried flower had the highest antioxidant property followed by 8.75g/L (T2) concentration and 6.25g /L (T3) concentration of *R. arboreum* flower kombucha. Among the three flavored kombucha, black grape-flavored kombucha had the highest phenolic content, followed by yellow berry-flavored kombucha and strawberry-flavored kombucha.

Table 4.1: Total phenolic content in different concentration of *Rhododendron* in kombucha

FERMENTATION DAYS	TEST 1	TEST 2	TEST3
7^{TH} DAY	0.0379 µgGAE/ml	0.04318 µgGAE/ml	0.05737 µgGAE/ml
14^{TH} DAY	0.1529 µgGAE/ml	0.1729 µgGAE/ml	0.1928 µgGAE/ml
21^{ST} DAY	0.2267 µgGAE/ml	0.2128 µgGAE/ml	0.2548 µgGAE/ml
30^{TH} DAY	0.3905 µgGAE/ml	0.4247 µgGAE/ml	0.5787 µgGAE/ml

Table 4.2: Total phenolic content in flavored kombucha.

FRUIT SAMPLES	Concentration µgGAE/ml
STRAWBERRY	0.5608 µgGAE/ml
YELLOW HIMALAYAN BERRY	0.8193 µgGAE/ml
BLACK GRAPES	1.1862 µgGAE/ml

4.2. Result of total flavonoid content

The table 4.3, indicate the flavonoid content measured on different days of fermentation process of kombucha process containing different concentration of dried flower of *R. arboreum*. The concentration of flavonoid content was measured in µgQE/ml (micrograms of quercetin equivalent per millilitre) for each samples. The concentration increases till 14^{th} day and then decreases as the fermentation process progressed to the 30 days. On 7^{th} day of fermentation, concentration observed to be highest in T2 (8.75 g/ml concentration of flower) with 1.5869 µgQE/ml followed by T3 (11.25g/ml concentration of flower) with 1.2613 µgQE/ml and lowest in T1 (6.25g/ml concentration of flower) with 0.8247 µgQE/ml. On 14th day of fermentation, the concentration of T1, T2 and T3 was 0.9934 µgQE/ml, 1.6036 µgQE/ml and 1.378 µgQE/ml respectively. On 21st day, there was decrease in flavonoid content compared to the 14th day. The concentration observed in T1, T2 and T3 was 0.871 µgQE/ml, 1.416 µgQE/ml and 1.0519 µgQE/ml respectively. Finally on the 30th day, the flavonoid concentration further decreased to 0.772 µgQE/ml, 1.2221 µgQE/ml and1.0147 µgQE/ml concentration in T1, T2 and T3 respectively.

The table 4.4 showed that the flavonoid content increased by addition of strawberry, yellow himalayan berry and black grapes in kombucha. The concentration increases to 2.0911 µgQE/ml, 3.0464 µgQE/ml and 2.0886 µgQE/ml in strawberry, yellow himalayan berry and black grapes respectively.

Overall the result showed that the flavonoid content of *R. arboreum* kombucha first increases and then decreases after the 14th day. Kombucha containing 8.75g /L concentration (T2) of *R. arboreum* dried flower had the highest flavonoid content followed by 11.25g/L (T3) concentration and 6.25g /L (T1) concentration of *R. arboreum* flower kombucha. Among the three flavors, yellow himalayan berry-flavored kombucha had the highest flavonoid content, followed by strawberry-flavored kombucha and black grapes-flavored kombucha.

Table 4.3: Total flavonoid content in different concentration of kombucha

FERMENTATION DAYS	TEST 1	TEST 2	TEST3
7TH DAY	0.8247 µgQE/ml	1.5869 µgQE/ml	1.2613 µgQE/ml
14TH DAY	0.9934 µgQE/ml	1.6036 µgQE/ml	1.378 µgQE/ml
21ST DAY	0.871 µgQE/ml	1.416 µgQE/ml	1.0519 µgQE/ml
30TH DAY	0.772 µgQE/ml	1.2221 µgQE/ml	1.0147 µgQE/ml

Table 4.4: Total flavonoid content in flavored kombucha.

FRUIT SAMPLES	CONCENTRATION µgQE/ml
STRAWBERRY	2.0911 µgQE/ml
YELLOW HIMALAYAN BERRY	3.0464 µgQE/ml
BLACK GRAPES	2.0886 µgQE/ml

4.3. Result of antioxidant property

The table 4.5 provides the result of percent inhibition in different concentrations of *R. arboreum* dried flower in kombucha analysed at different days of fermentation process. The percent inhibition was observed using DPPH assay. The percent inhibition increases till 14th day and then decreases as the fermentation process progressed to the 30 days. On 7th day of fermentation, percent inhibition observed to be highest in T2 (8.75 g/ml concentration of flower) with 70.89% followed by T3 (11.25g/ml concentration of flower) with 70.42% and lowest in T1 (6.25g/ml concentration of flower) with 69.95%. On 14th day of fermentation, the percent inhibition of T1, T2 and T3 was 72.42%, 75.42% and 73.36% respectively. On 21st day, there was decrease in percent inhibition compared to the 14th day. The percent inhibition observed in T1, T2 and T3 was 72.38%, 73.76% and 71.96% respectively. Finally on the 30th day, the flavonoid concentration further decreased to 72.32%, 72.76% and 70.96% inhibition in T1, T2 and T3 respectively.

The table 4.6 indicates that the addition of strawberry, yellow himalayan berry and black grapes fruits in kombucha increased its antioxidant property. It was observed that black grape flavored kombucha had the highest antioxidant property i.e. 77.42% inhibition followed by yellow himalayan berry had 76.79 % inhibition. Strawberry had lowest percent inhibition i.e. 75.81 %.

Overall the result showed that percent inhibition of *R. arboreum* kombucha first increases and then decreases after the 14th day. Kombucha containing 8.75g/L concentration (T2) of *R. arboreum* dried flower had the highest antioxidant property followed by 6.25g/L

(T1) concentration and 11.25g /L (T3) concentration of *R. arboreum* flower kombucha. Among the three fruit samples, black grape-flavored kombucha had the highest antioxidant activity, followed by yellow berry-flavored kombucha and strawberry-flavored kombucha.

Table 4.5: Percent inhibition of different concentrations of *R. arboreum* kombucha.

DAYS	TEST 1	TEST 2	TEST 3
7TH DAY	69.95%	70.89%	70.42%
14TH DAY	72.42%	75.42%	73.36%
21ST DAY	72.38%	73.76%	71.96%
30TH DAY	72.32%	72.76%	70.98%

Table 4.6: Percent inhibition of flavoured *R. arboreum* kombucha.

FRUITS	PERCENT INHIBITION
STRAWBERRY	75.81%
YELLOW HIMALAYAN BERRY	76.79%
BLACK GRAPES	77.42%

4.4. Result of antimicrobial activity

4.4.1. Result for *E. coli*

The figure 4.9 showed the result of antimicrobial activity of different concentrations of *Rhododendron* kombucha, 6.25g/L, 8.75g/L, and 11.25g/L respectively, against *Escherichia coli*. Positive control i.e. antibiotic showed an inhibition zone of 29mm and raw kombucha (RK) had an inhibition zone of 23mm. Amongst 3 different concentration of *Rhododendron* kombucha, T2 (8.75g/ml) had the highest inhibition zone of 24mm followed by T3 (11.25g/ml) inhibition zone of 20mm and the T1 (6.25g/ml) has the lowest inhibition zone of 19mm.

The figure 4.10 showed the antimicrobial activity of strawberry, black grapes and yellow himalayan berry flavored kombucha with the base *Rhododendron* kombucha of concentration 8.75g/ml flower against *E. coli*. Positive control i.e. antibiotic showed an inhibition zone of 29mm and raw kombucha (RK) had an inhibition zone of 23mm. Among the flavored kombucha black grapes (BG) had the highest zone of inhibition of 29mm followed by yellow berry (YB) with the inhibition zone of 21mm and strawberry (S) had the lowest inhibition zone of 18mm.

The result as shown in table 4.7 indicates that the black grape (BG) kombucha and positive control (i.e. antibiotic) had the highest and same inhibition zone of 29mm. The lowest inhibition zone of 18mm was observed by strawberry (S) flavored kombucha.

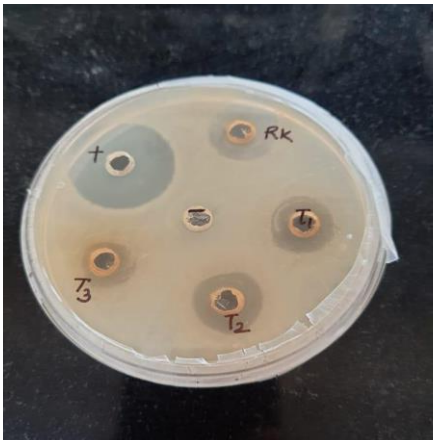

Fig 4.9: Inhibition zone of +ve control (antibiotic), -ve control (water), RK (raw kombucha), T1 (6.25g/ml *Rhododendron* flower kombucha), T2 (8.75g/ml *Rhododendron* flower kombucha), T3 (11.25g/ml *Rhododendron* flower kombucha) against *E. coli*.

Fig 4.10: Inhibition zone of +ve control (antibiotic), -ve control (water), RK (raw kombucha), S (Strawberry), YB (Yellow Himalayan Berry), BG (Black Grapes) against *E. coli.*

Table 4.7: Inhibition zone against *E. coli*

SAMPLES	INHIBITION ZONE
+ CONTROL	29 mm
- CONTROL	-
RK	23 mm
T1	19 mm
T2	24 mm
T3	20 mm
BG	29 mm
YB	21mm
S	18 mm

4.4.2. Result for *Bacillus subtilis*

The figure 4.11 showed the result of antimicrobial activity of different concentrations of *Rhododendron* kombucha, 6.25g/L, 8.75g/L, and 11.25g/L respectively, against *Bacillus subtilis*. Positive control i.e. antibiotic showed an inhibition zone of 29mm and raw kombucha (RK) had an inhibition zone of 20mm. Amongst 3 different concentration of

Rhododendron kombucha, T2 (8.75g/ml) had the highest inhibition zone of 16mm followed by T1 (6.25g/ml) inhibition zone of 15mm and the T3 (11.25g/ml) has the lowest inhibition zone of 13mm.

The figure 4.12 showed the antimicrobial activity of strawberry, black grapes and yellow himalayan berry flavored kombucha with the base *Rhododendron* kombucha of concentration 8.75g/ml flower against *B. subtilis.* Positive control i.e. antibiotic showed an inhibition zone of 29mm and raw kombucha (RK) had an inhibition zone of 20mm. Among the flavored kombucha black grapes (BG) had the highest zone of inhibition of 26mm followed by yellow berry (YB) and strawberry (S) had the same inhibition zone of 19mm.

The result as shown in the table 4.8 indicates that the positive control (i.e. antibiotic) had the highest inhibition zone of 29mm and if compared in the samples, black grapes (BG) had the highest inhibition zone of 26mm followed by yellow berry (YB) and strawberry (S) containing same inhibition zone of 19mm . The lowest inhibition zone of 13mm was observed by *Rhododendron* kombucha with concentration of 11.25g/ml (T3).

Fig 4.11: Inhibition zone of +ve control (antibiotic), -ve control (water), RK (raw kombucha), T1 (6.25g/ml *Rhododendron* flower kombucha), T2 (8.75g/ml *Rhododendron* flower kombucha), T3 (11.25g/ml *Rhododendron* flower kombucha against *B. subtilis.*

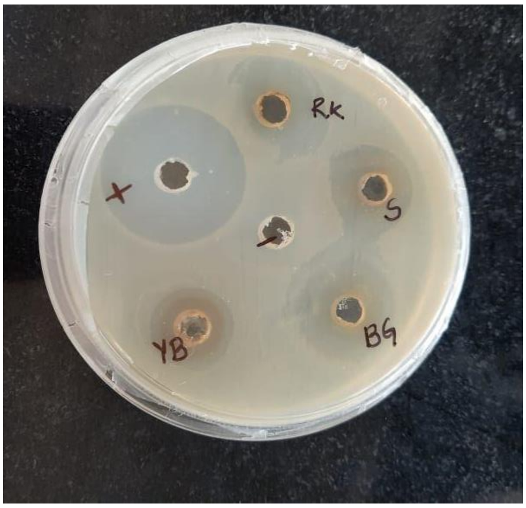

Fig 4.12: Inhibition zone of +ve control (antibiotic), -ve control (water), RK (raw kombucha), S (Strawberry), YB (Yellow Himalayan Berry), BG (Black Grapes) against *B. subtilis.*

Table 4.8: Inhibition zone against *B. subtilis*

SAMPLES	INHIBITION ZONE
+ CONTROL	29 mm
-CONTROL	-
RK	20 mm
T1	15 mm
T2	16 mm
T3	13 mm
BG	26 mm
YB	19 mm
S	19 mm

4.4.3. Result for *Salmonella typhi*

The result from the figure 4.13 obtained was the antimicrobial activity of different concentrations of *Rhododendron* kombucha, 6.25g/L, 8.75g/L, and 11.25g/L respectively, against *Salmonella typhi*. Positive control i.e. antibiotic showed an inhibition zone of 28mm and raw kombucha had an inhibition zone of 20mm. Amongst 3 different concentration of

Rhododendron kombucha, T2 (8.75g/ml) had the highest inhibition zone of 22mm followed by T1 (6.25g/ml) inhibition zone of 21mm and the T3 (11.25g/ml) has the lowest inhibition zone of 18mm.

The figure 4.14 showed the antimicrobial activity of strawberry, black grapes and yellow himalayan berry flavored kombucha with the base *Rhododendron* kombucha of concentration 8.75g/ml flower against *S. typhi*. Positive control i.e. antibiotic showed an inhibition zone of 28mm and raw kombucha had an inhibition zone of 20mm. Among the flavored kombucha black grapes (BG) had the highest zone of inhibition of 20mm followed by strawberry (S) with the inhibition zone of 18mm and yellow berry (YB) had the lowest inhibition zone of 17mm.

The result as shown in the table 4.9 indicates that the positive control (i.e. antibiotic) had the highest inhibition zone of 28mm and if compared in the samples, *Rhododendron* kombucha with the concentration of 8.75g/ml (T2) had the highest inhibition zone of 22mm followed by *Rhododendron* kombucha with the concentration of 6.25g/ml (T1). The lowest inhibition zone of 17mm was observed by yellow berry (YB) flavored kombucha.

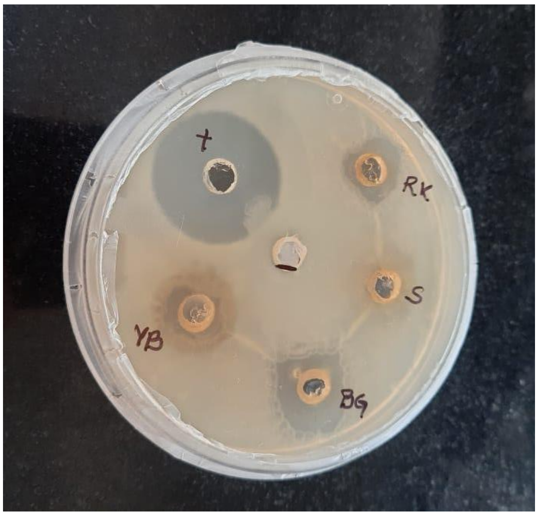

Fig 4.13: Inhibition zone of +ve control (antibiotic), -ve control (water), RK (raw kombucha), T1 (6.25g/ml *Rhododendron* flower kombucha), T2 (8.75g/ml *Rhododendron* flower kombucha), T3 (11.25g/ml *Rhododendron* flower kombucha) against *S. typhi*.

Fig 4.14: Inhibition zone of +ve control (antibiotic), -ve control (water), RK (raw kombucha), S (Strawberry), YB (Yellow Himalayan Berry), BG (Black Grapes) against *S. typhi*.

Table 4.9: Inhibition zone against *S. typhi*

SAMPLES	INHIBITION ZONE
+ CONTROL	28 mm
-CONTROL	-
RK	20 mm
T1	21 mm
T2	22 mm
T3	18 mm
BG	20 mm
YB	17 mm
S	18 mm

4.4.4. Result for *Staphylococcus aureus*

The results from the figure 4.15 indicate the antimicrobial activity of different concentrations of *Rhododendron* kombucha, 6.25g/L, 8.75g/L, and 11.25g/L respectively, against *Staphylococcus aureus*. Positive control i.e. antibiotic showed an inhibition zone of 19mm and raw kombucha had an inhibition zone of 19mm. Amongst 3 different concentration of *Rhododendron* kombucha, T2 (8.75g/ml) had the highest inhibition zone of

19mm followed by T3 (11.25g/ml) inhibition zone of 13mm and the T2 (6.25g/ml) has the lowest inhibition zone of 12mm.

The figure 4.16 showed the antimicrobial activity of strawberry, black grapes and yellow himalayan berry flavored kombucha with the base *Rhododendron* kombucha of concentration 8.75g/ml flower against *S. aureus.* Positive control i.e. antibiotic showed an inhibition zone of 19mm and raw kombucha (RK) had an inhibition zone of 19mm. Among the flavored kombucha yellow berry (YB) had the highest zone of inhibition of 19mm followed by black grapes (BG) with the inhibition zone of 12mm and strawberry (S) had the lowest inhibition zone of 9mm.

The result as shown in the table 4.10 indicates that the positive control (i.e. antibiotic), *Rhododendron* kombucha of concentration 8.75g/ml (T2), raw kombucha (RK) and yellow berry (YB) kombucha had the highest inhibition zone of 19mm. Followed by *Rhododendron* kombucha with the concentration of 11.25g/ml (T3). The lowest inhibition zone of 9mm was observed by strawberry (S) flavored kombucha.

Fig 4.15: Inhibition zone of +ve control (antibiotic), -ve control (water), RK (raw kombucha), T1 (6.25g/ml *Rhododendron* flower kombucha), T2 (8.75g/ml *Rhododendron* flower kombucha), T3 (11.25g/ml *Rhododendron* flower kombucha) against *S. aureus.*

Fig 4.16: Inhibition zone of +ve control (antibiotic), -ve control (water), RK (raw kombucha), S (Strawberry), YB (Yellow Himalayan Berry), BG (Black Grapes) against *S. aureus*.

Table 4.10: Inhibition zone against *S. aureus*

SAMPLES	INHIBITION ZONE
+ CONTROL	19 mm
-CONTROL	-
RK	19 mm
T1	12 mm
T2	19 mm
T3	13 mm
BG	12 mm
YB	19 mm
S	9 mm

The result shown in table 4.7, 4.8, 4.9 and 4.10 suggest that the *R. arboreum* kombucha containing 8.75g/L concentration (T2) of *R. arboreum* dried flower had the highest zone of inhibition followed by 6.25g/L (T1) concentration and 11.25g /L (T3) concentration. It exhibited significant antimicrobial activity against all four bacterial strains tested. The yellow himalayan berry-flavored kombucha had the highest zone of inhibition against *S. aureus*, while the black grapes-flavored kombucha had the highest zone of inhibition against *B. subtilis*, *S. typhi* and *E. coli*. The strawberry-flavored kombucha showed the lowest zone of inhibition against all four bacterial strains.

4.5 Result of sugar content

The table 4.11 indicate a decrease in sugar content during the fermentation process, suggesting that the sugar present at initial stage of kombucha were being consumed by microorganism involved in fermentation process. Sugar content on different days of fermentation process over time of 30 days. On the 7th day, the sugar content in T1, T2 and T3 were 4.6 °Brix, 4.9 °Brix and 5.2 °Brix respectively. On the 14th day, the sugar content in T1, T2 and T3 were 4.0 °Brix, 4.4 °Brix and 5.0 °Brix respectively. On the 21st day, the sugar content in T1, T2 and T3 were 3.5 °Brix, 3.6°Brix and 4.4 °Brix. Finally on the 30th day, the sugar content decreased to 2.9 °Brix, 3.1 °Brix and 3.8 °Brix in T1, T2 and T3 respectively.

The table 4.12 indicate increase in sugar content after addition of strawberry, yellow himalayan berry and black grapes fruits in kombucha. It was found that black grapes had the highest sugar content at 4.6 °Brix followed by strawberry had a sugar content of 3.6 °Brix and Yellow Himalayan Berry had 3.2 °Brix.

The results suggest that the sugar content of *R. arboreum* kombucha was significantly affected by the flavoring used. At the end of 1st fermentation kombucha containing 11.25g/L concentration (T3) of *R. arboreum* dried flower had the highest total sugar content followed by kombucha containing 8.75g/L concentration (T2) of *R. arboreum* dried flower. Among the flavored kombucha, the black grapes-flavored kombucha had the highest sugar content followed by strawberry flavored kombucha. The yellow himalayan berry-flavored kombucha had the lowest sugar content.

Table 4.11: Sugar content in different concentration of kombucha

FERMENTATION DAYS	TEST1	TEST2	TEST3
7^{TH} DAY	4.6 °Brix	4.9 °Brix	5.2 °Brix
14^{TH} DAY	4.0 °Brix	4.4 °Brix	5.0 °Brix
21^{ST} DAY	3.5 °Brix	3.6°Brix	4.4 °Brix
30^{TH} DAY	2.9 °Brix	3.1 °Brix	3.8 °Brix

Table 4.12: Sugar content in flavored kombucha.

FRUIT SAMPLES	SUGAR CONTENT (°Brix)
STRAWBERRY	3.6 °Brix
YELLOW HIMALAYAN BERRY	3.2 °Brix
BLACK GRAPES	4.6 °Brix

4.6. Result of pH

In the table 4.13 showed gradual decrease in pH, indicating increasing acidity in the kombucha. On the 7th day, T1 had a pH value of 3.48, T2 had a pH of 3.43, and T had a pH of 3.46. As the fermentation progressed, the pH values continued to decrease, with T1, T2, and T3 reaching pH values of 3.13, 3.14, and 3.19, respectively, by the 30th day.

In the table 4.14 showed the pH values of kombucha after flavoring. It was found that strawberry had a pH of 3.08, yellow himalayan berry had a pH of 3.07, and black grapes had the lowest pH value of 3.05.

The result indicate a gradual decrease in pH during the fermentation process of kombucha, suggesting the production of organic acids by the fermenting microorganisms. The kombucha containing 11.25g/L concentration (T3) of *R. arboreum* dried flower had the highest pH, which was significantly higher than all the flavored kombuchas tested. Among the flavored kombuchas, the strawberry-flavored kombucha had the highest pH, followed by yellow berry flavored kombucha. The black grapes-flavored kombucha had the lowest pH.

Table 4.13: pH in different concentration of kombucha

FERMENTATION DAYS	TEST1	TEST2	TEST3
7TH DAY	3.48	3.43	3.46
14TH DAY	3.35	3.32	3.34
21ST DAY	3.14	3.16	3.21
30TH DAY	3.13	3.14	3.19

Table 4.14: pH in flavored kombucha.

FRUIT SAMPLES	pH
STRAWBERRY	3.08
YELLOW HIMALAYAN BERRY	3.07
BLACK GRAPES	3.05

CHAPTER 5: CONCLUSION

In this thesis work the antioxidant activity, polyphenol content and antimicrobial activity of *Rhododendron* kombucha was examined. This kombucha was flavored with strawberry, yellow himalayan berry, and black grapes. The results showed that kombucha containing 8.75g/L concentration (T2) of *R. arboreum* dried flower had the maximum antioxidant activity, total flavonoid content and zone of inhibition against *S. aureus, B. subtilis, S. typhi, E. coli.* Whereas 11.25g/L concentration (T3) of *R. arboreum* dried flower had the highest total phenolic content and total sugar content. All three flavors of *Rhododendron* kombucha had antioxidant activity and contained polyphenols. The black grapes flavor had the highest antioxidant activity, total phenolic content and highest zone of inhibition against *B. subtilis, S. typhi* and *E. coli*, whereas yellow berry flavor had the highest flavonoid content. The strawberry flavored kombucha had the highest pH and yellow himalayan berry-flavored kombucha had the highest zone of inhibition against *S. aureus.* The findings of this study suggest that *Rhododendron* kombucha flavored with these fruits is a good source of antioxidants and polyphenols.

The results of this thesis are consistent with previous studies which suggested that kombucha is a beneficial source of antioxidants and polyphenols. The use of *Rhododendron* as a substrate for kombucha production is relatively new, and the addition of fruit flavors adds to the diversity of kombucha products available in the market. The results of this study suggest that *Rhododendron* kombucha flavored with strawberry, yellow berry, and black grapes may have potential as functional beverages and can be used to guide future research and product development in this field.

6. REFERENCES

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