

ANTIOXIDANT AND PHENOLIC EVALUATION OF FUNCTIONAL BEVERAGES KOMBUCHA INFUSED WITH OYSTER MUSHROOM (*PLEUROTUS OSTREATUS*) EXTRACT

Submitted in partial fulfilment of the requirements for the award of bachelor of
science degree in Microbiology

By

S HARINI SHREE (40300022)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
Accredited with Grade "A" by NAAC
JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119
MAY 2023



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

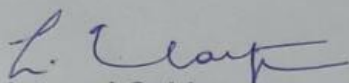
Accredited "A" Grade by NAAC | 12B Status by UGC | Approved by AICTE

www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **S. HARINI SHREE** (Reg No. 40300022) who carried out the project entitled "**ANTIOXIDANT AND PHENOLIC EVALUATION OF FUNCTIONAL BEVERAGES KOMBUCHA INFUSED WITH OYSTER MUSHROOM (PLEUROTUS OSTREATUS) EXTRACT**" under our supervision from January 2023 to May 2023.


Internal Guide

Dr.L . INBATHAMIZH


External Guide

Dr. JAYASINDHU MATHIYAZHAGAN



Head of the Department

Dr.V. RAMESH KUMAR

Submitted for Viva-voce Examination held on

Internal Examiner

External Examiner

DECLARATION

I S. HARINI SHREE (40300022) hereby declare that the Project Report entitled "ANTIOXIDANT AND PHENOLIC EVALUATION OF FUNCTIONAL BEVERAGES KOMBUCHA INFUSED WITH OYSTER MUSHROOM (*PLEUROTUS OSTREATUS*) EXTRACT" done by me under the guidance of Dr. L. Inbathamizh (Internal) and Dr. Jayasindhu Mathiyazhagan (External) in Centre for Laboratory Animal Technology and Research at Sathyabama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in **MICROBIOLOGY**.

DATE: 08 May 2023

PLACE: Chennai

S. Harini Shree

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of SATHYABAMA for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V. RAMESH KUMAR**, Head of the Department, School of Bio and Chemical Engineering, Department of Biotechnology for providing me with necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide **Dr. L. INBATHAMIZH** and **Dr. JAYASINDHU MATHIYAZHAGAN** for their valuable guidance, suggestions and constant encouragement that paved way for the successful completion of my project work.

I Thank **Dr. B. SHEELA RANI**, Director Research, Col. Dr. Jeppiaar Research Park to do project in Centre for Laboratory animal Technology and Research.

I Express my whole hearted thanks to **Dr. D. SARAVANAN**, **Dr. THIYAGARAJAN GOPAL** and **Dr. K. M. VENKATESH**, Faculty of CLATR, who supported whenever need clarification in my projects. I also thank Technicians, CLATR for their timely helped throughout my projects.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways in the successful completion of the project.

ABSTRACT

Kombucha is a popular fermented drink among many traditional fermented drinks. Kombucha is made from tea, sugar and SCOBY. Scoby (symbiotic culture of Bacteria and Yeast) is the inoculant which is a culture of bacteria and yeast. It is a slightly acidic, carbonated and low alcoholic refreshing drink. The steps involved in making kombucha starts from brewing tea. Then, sugar is added after preparing tea. Scoby is added (inoculant) at lukewarm temperature in a glass jar. Then, the jar is kept in a slightly warm and dark environment for fermentation around 7 - 21 days. As the culture increases in size, the flavour of the drink is enhanced. The microbes involved are Lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium*, *Bacillus cereus*, *Propionibacterium freudenreichii* and yeast of *saccharomyces* genus. *Pleurotus ostreatus* also known as oyster mushroom which is an edible mushroom. It belongs to the class Basidiomycetes and family Agaricaceae is popularly known as “dhingri” in India and grows naturally in the temperate and tropical forests on dead and decaying wooden logs or sometimes on dying trunks of deciduous or coniferous woods. It has high nutritional values and also has medicinal properties. It promotes heart and immune system health, encourages healthy blood sugar control and provides antioxidant and anti-inflammatory effects. Oyster mushroom water extract was prepared to be added to the kombucha. For the preparation of the jar, tea (15g), sugar (45g), water (2L), Scoby (along with kombucha starter culture) was taken. Equal amount tea is poured in two jars keeping one as test (Oyster mushroom extract infused kombucha) and another as control. Next equal weight of Scoby is added in both the jars. Then it is closed tightly and kept in warm and dark places. The test jar was infused with oyster mushroom and kept for the fermentation process. Then the sample is taken on the 0th day, 7th day, 14th day and given for the analysis. Also, we evaluated the applications of the oyster mushroom infused kombucha for its antimicrobial, and antioxidant activity. Overall, the oyster mushroom kombucha showed better antioxidant activities compared to the traditional kombucha.

KEY WORDS: Kombucha, oyster mushroom, fermentation, Antimicrobial, Antioxidant activity

INDEX

CHAPTER	TABLE OF CONTEXT	PAGE NO:
	Title	
	Abstract	
1	Introduction	1
1.1	Kombucha- an introduction	1
1.1.1	Origin of kombucha	1
1.1.2	Microbes involved in kombucha	1
1.1.3	Properties of kombucha	2
1.1.4	Health benefits of kombucha	2
1.2	Factors involving in antioxidant activity of kombucha	2
1.3	Introduction of oyster mushroom	3
2	Literature survey	5
2.1	Kombucha - an overview	5
2.2	Chemical composition of kombucha	5
2.3	Microbiological composition	7
2.3.1	Yeast	7
2.3.2	Bacteria	7
2.4	Green coffee kombucha	8
2.5	Green tea kombucha	9
2.6	Antimicrobial activity of kombucha	10
2.7	Antibacterial property of kombucha	11

2.8	Antioxidant activity of kombucha	12
2.9	Antioxidant activity of oyster mushroom	13
2.10	Volatile compounds profiling	14
2.11	Extraction of volatile compounds	16
2.12	Analysis of volatile compounds	16
2.13	Volatile determined in kombucha using HS-SPME GC-MS after the fermentation time of 6 days.	17
3	Aim and objectives	21
4	Materials and methodology	22
4.1	Cultivation of oyster mushroom	22
4.2	Mushroom powder extraction	25
4.2.1	Materials used	25
4.2.2	Methodology	25
4.2.2.1	Oyster mushroom extract powder	25
4.3	Preparation of kombucha	25
4.4	Collection of samples	26
4.5	Antimicrobial test	26
4.5.1	Materials required	26
4.5.2	Procedure	27
4.6	pH test	27

4.7	Antioxidant test	27
4.7.1	Materials required	28
4.7.2	Procedure	28
4.8	Phenolic test	29
4.8.1	Materials required	29
4.8.2	Methods	29
5	Results and discussion	31
5.1	Mushroom cultivation	31
5.2	Yield	31
5.3	Oyster mushroom extract powder	32
5.4	Antimicrobial test	32
5.5	pH test	34
5.6	Antioxidant test	36
5.7	Phenolic test	40
6	Summary and conclusion	45
7	References	46

LIST OF TABLES

TABLE NO.	CONTENT	PAGE NO.
Table 2.2	General chemical composition of kombucha	5
Table 2.10.1	Chemicals used for three groups listed as type 1, type 2, type 3	15
Table 2.13	Volatile compounds present in kombucha determined through HS-SPME/ GC-MS	17
Table 5.4.1	Results for antimicrobial test done with mushroom infused kombucha samples against traditional kombucha	34
Table 5.6.1	Table showing readings from spectrophotometry	36
Table 5.6.2	Table showing average value of DPPH assay from spectrophotometry	37
Table 5.6.3	Table showing final RSA% of samples	37
Table 5.7.1	Table showing the readings of phenolics test	40
Table 5.7.2	Table showing average value of phenolics test	41
Table 5.7.3	Table showing RSA% of phenolics test	42

LIST OF FIGURES

FIGURE NO.	CONTENT	PAGE NO.
Fig 5.2.1	Mushroom grown in bags	31
Fig 5.3.1	Mushroom extract powder	32
Fig 5.4.1	Plate of antimicrobial test showing zone of inhibition	32
Fig 5.5.1	pH reading of 0 th day control	35
Fig 5.5.2	pH reading of 0 th day test	35
Fig 5.5.3	pH reading of 7 th day control	35
Fig 5.5.4	pH reading of 7 th day test	35
Fig 5.5.5	pH reading of 14 th day control	35
Fig 5.5.6	pH reading of 14 th day test	35
Fig 5.6.1	DPPH assay on 96-well plate	37
Fig 5.6.2	Graph showing antioxidant activity of kombucha on 0 th day	39
Fig 5.6.3	Graph showing antioxidant activity of kombucha on 7 th day	39
Fig 5.6.4	Graph showing antioxidant activity of kombucha on 14 th day	40
Fig 5.7.1	Phenolics test on 96-well plate	41
Fig 5.7.2	Graph showing the total phenolic content of kombucha on 0 th day	43
Fig 5.7.3	Graph showing the total phenolic content of kombucha on 7 th day	44
Fig 5.7.4	Graph showing the total phenolic content of kombucha on 14 th day	44

LIST OF ABBREVIATION:

- GCMS - Gas chromatography Mass spectrometry
- OM - Oyster Mushroom
- SCOBY - Symbiotic culture of bacteria and yeast
- RSA% - Radical Scavenging Activity
- pH - Potential of hydrogen
- DPPH - Diphenyl picryhydrazyl
- LAB - Lactic acid bacteria
- GI - Gastro intestinal
- UV - ultra violet
- 0C - 0th day control
- 0T - 0th day test
- 7C - 7th day control
- 7T - 7th day test
- 14C - 14th day control
- 14T - 14th day test
- *P. ostreatus* - *Pleurotus ostreatus*
- AAB - acetic acid bacteria
- DNA - Deoxyribonucleic acid
- LDH - Lactic dehydrogenase
- ROS - Reaction oxygen species
- ABTS - Azino-bis-ethylbenzthiazoline sulfonic acid
- FRAP - Ferric iron-reducing antioxidant power
- SPME - Solid-phase micro extraction
- PCA - Principal component analysis
- FC - Folin - ciocalteu Reagent
- OD - Optical Density

CHAPTER 1 INTRODUCTION

1.1 KOMBUCHA - AN INTRODUCTION:

Kombucha is a fermented drink made from tea, sugar and **scoby**. Scoby is the inoculant which is a culture of bacteria and yeast. The bacteria used is from acetobacteraceae family and the osmophilic yeast. This scoby is also known as Manchurian mushroom. The steps involved in making kombucha starts from brewing tea. Sugar is added after the tea is made. Then the scoby [symbiotic culture of bacteria and yeast] inoculant is added at lukewarm temperature, in a glass jar and the jar is kept in slightly warm and dark environment for fermentation for around 14 days(Zofia *et al.*, 2020). The increase in size of scoby occurs. This happens when the organism thrives and ferments the sugar. So, the process happening here is sugar fermentation. As the culture increases in size, the flavor of the drink is enhanced (zenwebnet.admin|jan4,2020).

1.1.1 ORIGIN OF KOMBUCHA:

Kombucha is originated in China, through traditional processing. Then it was disseminated across the world. It is a slightly acidic, carbonated[fizzy], low alcoholic refreshing drink. It has lots of probiotic organisms. As the fermentation time period is increased, the flavors and bioactive compounds like polyphenols, vitamin B group, vitamin C group and minerals are enhanced. Minerals like Iron (Fe), Manganese (Mn), Zinc (Zn), Copper (Cu), Nickel (Ni) are enhanced(Batista *et al.*, 2022)

1.1.2 MICROBES INVOLVED IN KOMBUCHA:

The microbes involved are Lactic acid bacteria (LAB)- lactobacillus, Bifidobacterium, Bacillus cereus, Propionibacterium freudenreichii and yeast of saccharomyces genus.(Batista *et al.*, 2022)

1.1.3 PROPERTIES OF KOMBUCHA:

1. The polyphenols are responsible for the antioxidant property and anti-inflammatory properties. Also, the low pH and bacterial cellulose found in Scoby, inhibits bacterial action on skin and stimulates skin regeneration.
2. Scoby is also used in absorbing heavy metals and for the textile industry.
3. Kombucha is found to have properties to work against human breast cancer, colon cancer. The result showed that kombucha inhibits the growth and proliferative properties of cancerous cells.
4. Organic acids such as Acetic acid, Gluconic acid, Glucuronic acid are found in kombucha.(Batista *et al.*, 2022)

1.1.4 HEALTH BENEFITS OF KOMBUCHA:

These organisms help in healthy microbiome but also produces relevant compounds such as amino acids, organic acids which increases its acidity. Hence, a recommended fermentation time is advised. Kombucha has many useful properties as studied under in vivo, invitro environments, such as antioxidant, anti-inflammatory, anti-bacterial, anti-diabetic, anti-carcinogenic activities. It also helps in reducing the levels of cholesterol(anti-hypercholesterolemia) and improving liver, Gastro intestinal (GI), immune metabolism, hepatoprotective properties (Batista *et al.*, 2022).

1.2 FACTORS INVOLVED IN ANTIOXIDANT ACTIVITY OF KOMBUCHA:

The antioxidant property depends on three important factors;

1. The type and composition of tea infusion,
2. The Scoby
3. The Fermentation process.

1.3 INTRODUCTION OF OYSTER MUSHROOM:

One of the most widely cultivated and used mushrooms is oyster mushroom (*Pleurotus ostreatus*). It belongs to,

Kingdom : Fungi

Division : Basidiomycetes

Class : Agaricomycetes

Order : Agaricales

Family : Pleurotaceae

Genus : *Pleurotus*

Species : *P. Ostreatus*

Vitamin D deficiency is a rapidly increasing worldwide problem in humans due to changing lifestyle, poor exposure to sunlight, usage of products like sunscreen, obesity, melanin etc., are the major reasons for insufficient vitamin D in humans naturally. Deprivation of vitamin D can cause major health risks such as cancer, rickets, osteoporosis, diabetes, cardiovascular disease and autoimmune disease (Wu & Ahn, 2014). It is reported and estimated that around 30-50% of individuals in Europe and US are suffering from deficiency of vitamin D. In Africa, an article mentioned that around 80% of the population is facing vitamin D deficiency. There are two distinct forms of vitamin D occurring in nature namely, Vitamin D₂ and Vitamin D₃. Vitamin D₂ is known as Ergocalciferol. This is synthesized from Ergosterol when it is activated by UV irradiations, since Ergosterol is the precursor of Vitamin D₂ (Keflie *et al.*, 2019).

Vitamin D₃ is known as cholecalciferol exist in the products of the animals like fish liver oil, butter, margarine etc. Vitamin D₂ can be environmentally obtained from yeast and mushrooms. Hence, the very few sources of vitamins from nature apart from sunlight are from mushrooms, fish, yeast and egg yolk. (Keflie *et al.*, 2019) It is a problematic scenario when vegetarians are recommended to consume uptake vitamin D₂. Since, they are majorly derived from animals so far. But mushrooms could be a very potential and effective source of vitamin D and could be the best alternative (Wu & Ahn, 2014). Oyster mushrooms [*pleurotus ostreatus*] and other mushroom species are some of the ideal considerations for the source of vitamin D (Keflie *et al.*, 2019).

Though cultivated mushroom is rich in ergosterol they do not contain significant amount of vitamin D but they can be triggered by exposure to UV radiations. Studies suggested and proved that UV irradiations, along with other parameters such as

temperature, exposure time, intensity of radiation etc., increased the synthesis of vitamin D₂ under controlled cultivation of mushrooms (Wu & Ahn, 2014). Ergosterol is the precursor of vitamin D₂, in mushrooms. The formation of ergocalciferol from ergosterol occurs instantly after the exposure to UVB light. Since the UVB rays are not able to penetrate deeply into the mushroom, only the superficially available ergosterol are up and the process is slowly leased. Hence the yield of ergocalciferol is limited. But also, prolonged exposure to sunlight or other UVB radiation leads to the formation of dimer compounds and other unwanted products (Keflie *et al.*, 2019).

CHAPTER 2

REVIEW OF LITERATURE

2.1 KOMBUCHA - AN OVERVIEW:

Kombucha is a popular fermented drink among many traditional fermented foods. Bacteria and yeasts present in the medium helps for inhibiting the growth of microorganisms and creates a powerful symbiosis during the growth of microorganisms (Vitas *et al.*, 2013). These are two phases:

1. A floating biofilm and,
2. sour liquid phase.

The main components in the liquid are acidic acid, gluconic acid and ethanol(Czaja *et al.*, 2006)

Kombucha is able to convert sugar and tea in a period from 7 to 10 days in a slightly sour, lightly carbonated and refreshing drink which are composed of several acids, 14 amino acids, vitamins and some hydrolytic enzymes under aerobic conditions.(Vitas *et al.*, 2013)

2.2 CHEMICAL COMPOSITION:

Table 2.2 general chemical composition of kombucha(Villarreal-Soto *et al.*, 2018a)

	Compound	Initial sucrose	Form time(days)	Reference
Organic acids	Acetic acid	70g/L	15D	Blanc (1996)
	Gluconic acid	100g/L	60D	Chen and Lin (2000)
	Glucuronic acid	70g/L	21D	Loncar <i>et al.</i> , (2006)
	Lactic acid	100g/L	18D	Jayabalan <i>et al.</i> , (2007)
Vitamins	Vit B1	70g/L	15D	Bauer-petrovska& petrushevsk -tozi (2000)
	Vit B2	70g/L	10D	Malbasa <i>et al</i> (2011)

	Vit B6	70g/L	15D	Bauer-petrovska& petrushevska (2000)
	Vit B12	70g/L	15D	Petrushevska-tozi
	Vit C	70g/L	10D	Malbasa <i>et al</i> (2011)
General composites	Ethanol	100g/L	20D	Chen & Liu (2000)
	Proteins	100g/L	12D	Jayabalan <i>et al</i> (2007)
	Tea polyphenols	100g/L	15D	Chu & chen (2006)
Minerals	Cu, Fe, Mn, Ni, Zn	70g/L	15D	Bauer-petrovska & Petrushevska -tozi (2000)
Anions	F, Cl, Br, I, NO ₃ , Hpo ₄ , So ₄	100g/L	7D	Kumar, Narayan, & Hassarajani (2008)

The composition and properties of kombucha tea is important for better understanding in its kinetics (Nguyen *et al.*, 2015). Final concentration of sugar can differ from one fermentation to another fermentation which shows that metabolic pathways do not occur in the same way (Chen & Liu, 2000). The chemical composition and the concentration of each metabolic produced in kombucha will depend on the initial sugar concentration and the inoculum. Glucuronic acid is considered to be the main agent among the main constituents of kombucha tea. After the completion of fermentation process, the products mainly formed in kombucha are ethanol, gluconic acid, and acetic acid. It also contains tea polyphenols (catechins, theaflavins and flavanols) since, the substrate in tea. Also, organic acids are present (e.g.; folic, carbonic, lactic, malic, oxalic, malonic, tartaric, succinic, glucuronic, pyruvic, citric, usnic acid). It also contains water soluble vitamins such as B1, B2, B3, B6, B12 and C as well as vitamin E enzymes (amylase, invertase). In recent study, it was observed that the ratio of gluconic acid changes by using the synthetic microbial community (SMC) and the amount of gluconic acid present in kombucha was related to the taste quality. (Kumar & Joshi, 2016)

2.3 MICROBIOLOGICAL COMPOSITION:

SCOBY is the inoculant used in the fermentation process of kombucha. SCOBY is the culture of bacteria and yeast [symbiotic association of bacteria and yeast] (Villarreal-Soto *et al.*, 2018a)

2.3.1 Yeast:

Most of the yeast species ferments sugar to ethanol. Most commonly used yeasts for the modern alcoholic fermentation being *Saccharomyces cerevisiae* because it has high efficiency. (Lopez *et al.*, 2014) In kombucha culture many yeast genus are present and has been reported including species of *Zygosaccharomyces*, *saccharomyces*, *lachancia*, *torulaspora*, *pichia*, *saccharomycoides*, *schizosaccharomyces*, *candida*, *kloeucera/hanseniaspora*, *Brettanomyces/dekkera* and *kluveromyces*. (Chakravorty *et al.*, 2016) (Coton *et al.*, 2017) (Marsh *et al.*, 2014) In the kombucha tea culture, some other yeasts were present they are *Zygosaccharomyces* as the predominant yeast with 84.1% of relative percent of abundance, *dekkera* and *pichia* species are present with the percentage of 6% and 5% respectively (Watawana *et al.*, 2016)

2.3.2 Bacteria:

In kombucha tea culture the dominant bacteria are AAB, which are aerobic bacteria in which substrates are used as alcohol to form acetic acid. Bacteria needs large amount of oxygen for their activity and growth. The metabolic process is based on the conversion of acetaldehyde hydrate into acetic acid and acetaldehyde into ethanol by using the enzyme named acetaldehyde dehydrogenase (Jayabalan *et al.*, 2007). Any acetic acid bacteria [AAB] are present in the tea fungus includes *Acetobacter xylinoides*, *Bacterium gluconium*, *Acetobacter aceti*, *Gluconobacter oxydans* and *Acetobacter pasteurianus* (Jayabalan *et al.*, 2014) (Watawana *et al.*, 2016) It was identified from the work of (Marsh *et al.*, 2014) that the rRNA sequence analysis showed around 86% to 99% relative abundance of gluconic acetobacter by all fermentation in both biofilm and liquid medium. Similar results were reported by (Watawana *et al.*, 2016) found that gluconacetobacter as the main one with a relative percent of 85.6 by fermenting coconut water with the tea fungus and some bacteria species like *Acetobacter*, *Lactobacillus*, *Leuconostoc* and *Bifidobacterium* was seen in the less proportion.

2.4 GREEN COFFEE KOMBUCHA

Apart from tea, one of the most consumed popular beverages around the world is coffee, including green coffee. In coffee extract, the biological active substance is present which includes two groups of compounds. The first are substances with antioxidant activity such as polyphenols. Polyphenols can neutralize various forms of superoxide free radicals. For this reason, polyphenols are valuable in protecting the DNA of skin cells. The second group are purine alkaloids, in that caffeine and trigonelline are the most important. These compounds stimulate metabolism and improve cell oxygenation and microcirculation in our body (Roshan *et al.*, 2018) (Mukkundur Vasudevaiah *et al.*, 2017a) (Fukagawa *et al.*, 2017). The preparation of infusions from green coffee is similar to the preparation of tea, where SCOBY was used for the fermentation process and it contributes many valuable properties (Watawana *et al.*, 2015). Therefore, the main purpose of this work is to determine the properties of ferments present in green coffee beans after fermentation process. For this purpose, the structure of prepared ferments was evaluated by using a method of Chromatographic and a spectrophotometric determining the scavenging of extrinsic and intracellular free radicals as well as estimating of superoxide dismutase activity. The capability of inhibiting the activity of matrix metalloproteinases, collagenase and elastase which plays an important role in skin aging process was determined to assess anti-aging properties. Cytotoxicity tests (Alamar Blue, Neutral red and lactic dehydrogenase (LDH)) were also done on skin cells, Keratinocytes and fibroblasts for evaluating cytotoxicity of ferments prepared. Effect of fermentation time on Antioxidant and Anti-ageing properties of green coffee Kombucha ferments (Mukkundur Vasudevaiah *et al.*, 2017b)

In plants, the basic active ingredients are polyphenols and flavonoids, which are responsible for their antioxidant activity by neutralizing free radicals that may produce oxidative stress. Green coffee is a rich source of polyphenols and flavonoids and green coffee beans contain 6-10% of polyphenols depending on the origin. Chlorogenic acids are the main polyphenols compounds present in green coffee beans, and they are present in about 90% of the concentration of total phenolic content. Furthermore, Green coffee also contains a high concentration of caffeine derivatives ranging from 1% to 5% (Farah, 2012) (Perdani & Pranowo, 2019). The most important bioactive compounds of coffee are phenolic compounds such as chlorogenic

acids and its derivatives, alkaloids from methylxanthine group (caffeine), nicotinic acid (Vit B3) and its precursor trigonelline, magnesium and potassium (Gómez-Ruiz *et al.*, 2007). In coffee bean extract the main groups of phenolic compounds are chlorogenic acids (CG4), Which are esters of quinic acid and trans-hydroxy cinnamic acid. The green coffee extract was regarded as raw materials for the preparation of kombucha. At the first stage of the research, the antioxidant potential of the tested green coffee extract and kombucha ferments was estimated. It was shown that the assessed material possesses antioxidant potential and the percentage of free radicals scavenged by the tested extract at the process of fermentation. The antioxidant potential was high in the case of all analyzed extracts. Green coffee extract identified the highest antioxidant potential. The fermentation process of green coffee extract was done upto 28 days and was also obtained by strong antioxidant properties, after 30 mins of measurements, it had 14% lower antioxidant potential compared to the results estimated for the green coffee extract during fermentation process. The biggest difference was identified between the extract and the green extract subjected to a fermentation process of 7 days. The green coffee extract showed 120% higher potential than the ferments seen after 7 days during the process of fermentation. In the case of tea fermentation with an increase in the antioxidant potential with an increase in fermentation time was also observed (Butt *et al.*, 2011) (Czekanska, 2011; Finkel & Holbrook, 2000; Floegel *et al.*, 2011; Jayabalan *et al.*, 2007, 2014; Nakayama, 1997; O'brien *et al.*, 2000; Repetto & Sanz, 1993; Sen *et al.*, 2010) The conducted research shows that during long-term fermentation the enzymes released by bacteria are efficient in scavenging nitrogen and superoxide radicals and poor efficiency in scavenging hydroxyl radicals (Chu & Chen, 2006; Finkel & Holbrook, 2000; Jayabalan *et al.*, 2014)

2.5 GREEN TEA KOMBUCHA

Green tea has definite astringency, bitter taste, and green flavors due to its post-harvest treatment without withering and enzymatic oxidation. The volatile profile is determined by headspace-solid phase microextraction along with gas chromatography mass spectrometry. Polyphenol profile was determined by using high-performance liquid chromatography equipped with ion trap mass spectrometry, and the result indicated that the amount of most polyphenols in green tea infusion decreased after fermentation. The antioxidant activity of fermented tea infusion was decreased, especially the DPPH- free-radical-scavenging ability and the ferric

reducing power. Green tea is more popular in most of Asia, mainly in China, and Japan because bioactive compounds like polyphenols was present and then black tea which have more benefits.(Su *et al.*, 2022)

2.6 ANTIMICROBIAL ACTIVITY OF KOMBUCHA:

It was reported that kombucha has an extreme antimicrobial effect on a wide range of microorganisms(Vohra *et al.*, 2019a). Further, many estimations have been evaluated to show the inhibitory performance against abundance of microorganisms including gram-negative and gram-positive bacteria(Dufresne & Farnworth, 2000). Therefore, kombucha is able to prevent the growth of a huge range of microorganisms like *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, *Helicobacter coli* (causative organisms for common diarrhea), *Yersinia enterocolitica*, *Candida albicans*, *Shigella sonnei*, *Campylobacter jejuni* and *Staphylococcus aureus*. Even Though due to the presence of acetic acid in the scoby present in kombucha shows the antimicrobial effect and also low ranges of pH can be favorable (Podolich *et al.*, 2019).During the fermentation process of kombucha, the proteins and catechins and some components were considered as critical factors for the improvement of antimicrobial properties. It was confirmed that both acetic acid and catechins found in kombucha have bactericidal properties and it was described that kombucha have bactericidal properties and it was described that kombucha has antibiotic substance that led to an increase in the antimicrobial activities. In recent times, due to the high-quality antimicrobial activity of kombucha, it can be broadly utilized as an agent to reduce the pathogens associated with human illness. The researchers noted that the preparation process of kombucha and fermentation time of kombucha also the origins could affect the antimicrobial activity of kombucha. To increase the antimicrobial properties of some substance. Kombucha can be utilized as a supplement and get better results(St-Pierre, 2019).

2.7 ANTIBACTERIAL PROPERTY OF KOMBUCHA:

One of the most predominant polyphenols found in tea is catechin which has a high potential of antibacterial properties. Major antibacterial compounds of kombucha have been identified as polyphenols of catechin and isorhamnetin which belong to the flavan-3-ol and flavonol classes of flavonoids, respectively (Bhattacharya *et al.*, 2016). Due to the presence of hydroxylation at position 5 and 7 of the A ring and position 3

of the C ring provide the antibacterial activity of these polyphenolic compounds. The free hydroxyl groups in the B ring of the flavonoids are also responsible for the antibacterial activity (Cushnie & Lamb, 2011; Rojas *et al.*, 1992). Bacteria have an important role in antimicrobial activity of kombucha by producing bacteriocins, enzymes and other protein compounds. Proteinaceous compounds such as bacteriocins show bactericidal activity against species which were closely related to the producer strain (Sezer & Güven, 2009). Due to the difference in the chemical compounds of black tea, the pH value differs (Battikh *et al.*, 2012). Furthermore, it was stated that the reduction in pH can be caused due to the formation of organic acid by acetic acid bacteria and the inter-connection of minerals and herbal compounds of tea and fungus (Battikh *et al.*, 2012; Malbaša *et al.*, 2011; Talawat *et al.*, 2006). Also, the concentration of sugar had an important effect on antibacterial activity of kombucha beverages and most of the antibacterial activity was observed in beverages prepared with a 8% concentration of sugar, it causes elevated organic acid production and also more antimicrobial activity (Santos Jr *et al.*, 2009). In tea, the sugar is metabolized into fructose and glucose by the microorganisms in kombucha. Firstly, glucose was converted into ethanol as CO₂ by yeasts and then ethanol was converted into acetic acid and other organic acid by acetic acid bacteria. In kombucha beverages, the microorganisms are needed for the nutrition source i.e., sugar and it was summarized that the increase in antibacterial activity was associated with the increase in the concentration of sugar (Battikh *et al.*, 2012; Jayabalan *et al.*, 2014; Mo *et al.*, 2008).

Fermentation time had a crucial effect on the antibacterial activity of kombucha beverages. Generally, during the 21 days fermentation time all the tested fermented beverages showed the most antibacterial activity. In several research studies, it was reported that increased antibacterial activity of kombucha beverages along with the increase in fermentation time showed better result (Battikh *et al.*, 2012; Vohra *et al.*, 2019b). During fermentation time, it affects the compounds of kombucha beverages and its biological activities including the antibacterial activity (Wolfe & Dutton, 2015). (Chu & Chen, 2006) Even though, depending on the type of bacteria at different fermentation times, the antibacterial activity also occurs (Villarreal-Soto *et al.*, 2018b).

2.8 ANTIOXIDANT ACTIVITY OF KOMBUCHA:

Antioxidant activity can be examined in three forms like inhibition properties of molecules, scavenging of pro-oxidant metals and binding of prooxidant metals. Therefore, it was proven that these effects of antioxidant activity work on several diseases like diabetes and cancer (Chu & Chen, 2006). The first step of the antioxidant activity is removing the free radicals which were produced in the oxidation reaction. As we know free radicals are not beneficial for the human body because they are able to initiate the multiple chain reaction and at last these reactions lead to harm to cells or even death (Shebis *et al.*, 2013). The status of oxidative stress can be determined by the balance between the cellular ROS generation and antioxidant deficiency capacity (Tang *et al.*, 2019). In kombucha's fermentation several beneficial compounds are seen with radical inhibiting activities and useful antioxidant agents are found although kombucha's fermentation process does not pose a complicated pathway (Ivanišová *et al.*, 2019; Nazemi *et al.*, 2019). In kombucha beverages, two primary classes of compounds are present such as catechins and polyphenols. Polyphenols are extremely good compounds that have been identified in kombucha and can effectively scavenge free radicals from reactive oxygen species (ROS) which is the main purpose for introducing kombucha as a potent antioxidant agent. Polyphenols show different structures, of which tannins, phenolic acids, flavonoids and stilbenes are recognized as the primary structure of dietary polyphenols. Records from various examinations have given the relations between the intake of polyphenols and reduced the circumstance of several human illnesses and also verified their function in the prohibition of cancer, neuro degenerative disorder, obesity and cardiovascular. Specific polyphenols can be exhibited in the shape of polymers, glycosides or esters; therefore, they are not able to be taken in their normal forms. Before absorption, they should be hydrolyzed using intestinal enzymes or also can be hydrolyzed even by the colonic microflora. Certainly, polyphenols tolerate different certification mechanisms like sulfation, methylation and glucuronidation initially in the intestinal cells and then within the liver (D'Archivio *et al.*, 2007).

It has been reported that the presence of complex phenolic compounds in an acidic circumstance or even the discharge of various species of enzymes released by fungi and bacteria can lead to the splitting of large and complex molecules into smaller and simpler molecules, which leads to an increase in phenolic compounds in kombucha.

Researchers examined the fermentation time of kombucha and its origins on its antioxidant activities by in vitro free radical scavenging assays and they summarized that with increasing the fermentation time of kombucha, the antioxidant activity has a direct relationship with origin materials and the culture time of kombucha in turn described the metabolites types itself (Greenwalt *et al.*, 2000).

Antioxidant activities was done act by three assays,

(i) 2, 2'-azino-bis-3-ethylbenzthiazoline-sulfonic acid (ABTS) free radical-scavenging rate.

(ii) 2, 2 - diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging rate.

(iii) Ferric iron - reducing antioxidant power (FRAP).

◆DPPH is a stable free radical molecule of red color. (Gómez-Ruiz *et al.*, 2007)

2.9 ANTIOXIDANT ACTIVITY OF OYSTER MUSHROOM:

Mushrooms have a protective role just like many plants. They produce Phyto compounds with antioxidant activity which potentially protects organisms against oxidative stress and also it prevents the formation of free radicals in diseases due to the reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Avcı *et al.*, 2014). Furthermore, researchers have reported that oyster mushrooms have radical scavenging activity. Hence, the consumption of oyster mushrooms protects the human body (Wong *et al.*, 2009).

Therefore, mushroom acts as a functional food and as a source for the production of pharmaceuticals and nutraceuticals due to their antioxidant, anticancer and antibacterial properties (Barros *et al.*, 2008; Lo & Cheung, 2005). The phenolic compounds, polyketides, terpenes, variegated acid, diboviquinone and steroids are the major secondary metabolites that mushrooms accumulate. (Cheung *et al.*, 2003)

Due to the presence of phenolic compounds, mushrooms have the majority of their antioxidative properties which allows them to operate as reducing agents, singlet oxygen quenchers and hydrogen donors (Rice-Evans *et al.*, 1997). According to the tests, *Pleurotus ostreatus* extract showed higher phenolic content. (Nwachukwu & Uzoeto, 2010)

2.10 VOLATILE COMPONENTS PROFILING:

◆According to the odor characteristics of volatile components, it was roughly classified into seven types, they are namely, herbal, green, floral, fruity, sweetish, tasty and other.

◆Volatile profile was mainly composed of (2)-3-hexen-1-ol and linalool oxide upto 1 day fermentation, it turned to be linalool, geraniol, and methyl salicylate and other substances in few days (Su *et al.*, 2022).

◆Volatile components was further divided into three types of groups;

(i) Experimental groups,

(ii) Significant difference,

(iii) Significantly higher experimental group.

□Type 1 has 28 substances of which 12 chemicals were added.

□Type 2 consisted of 12 substances of which 12 chemicals were added.

□Type 3 consisted of 32 substances of which 12 chemicals were disappearing after the process of fermentation.

Table 2.10.1 table showing chemicals used for three groups listed as type 1, type 2, type 3

TYPE 1 GROUP	TYPE 2 GROUP	TYPE 3 GROUP
Methyl benzoate Citral (R)- Citronellol Methyl salicylate Nerol Dihydroactini-diolide (Z)-3-hexen-1-ol Benzaldehyde □-terpineol Linalool Geraniol □-damascenone Phenethyl alcohol	Geranylacetone Cis-jasmone Phenol	□-ionone (Z)- Z-penten-1-ol Methyl-heptadienone Hexanoic acid 2-furanmethanol 1-hexanol

Furthermore, the amount of Benzyl alcohol, 1-betanol, theapirane, indole and other substances were reduced after fermentation(Su *et al.*, 2022)

This experiment was done to study the effects of fermentation on antioxidant activity. Both DPPH inhibition kinetics and orthodox quantification of inhibition percentage were carried out. The rate of microbial metabolism represents the antioxidant activity.

It also represents equivalent changes in the antioxidant activity. So the increased rate of antioxidant activity shows increased rate of microbial function. The inhibition was calculated by using the formula,(Zhao *et al.*, 2019)

DPPH radical scavenging activity

$$= \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100\%.$$

2.11 EXTRACTION OF VOLATILE COMPOUNDS:

After the headspace compounds were extracted the kombucha's volatile compounds were determined and concentrated using solid-phase micro extraction (SPME). Extraction of compounds was performed with a 50/30 μm DVB/CAR/PDMS fiber and manual holder. Aliquots (2ml) sample of the kombucha were transferred to 10ml vials which were tightly closed (hermetically) with an equilibrium time of 30 mins. The SPME was operated at 60°C and an extraction time of 15 mins. (Ribeiro *et al.*, 2017)

2.12 ANALYSIS OF VOLATILE COMPOUNDS:

Analysis of volatile compounds were evaluated as described by (Ribeiro *et al.*, 2017) with some modifications. Thermal desorption was performed after the extraction in the GC injector at 240°C for 5 minutes. The analyses were performed using a GC-MSQP2010 SE system (shimadzu) equipped with a DB - WAX column (30m $\times$ 0.25 mm i.e. $\times$ 0.25 μm at 50°C for 5 min initially; then, the temperature was increased from 3°C/min upto 190°C for 10 minutes. A carrier gas was used called High-purity helium with a constant flow of 1.2 mL/min. In spitless mode the injection were performed with an injection time of 2 minutes. After 3 minutes, from 45 to 1,000 m/z Mass-spectra were acquired continuously. The temperature of ion source was 230°C, and the temperature of quadrupole's temperature was 150°C. (Zhang *et al.*, 2021)

2.13 VOLATILE COMPONENTS PRESENT IN KOMBUCHA DETERMINED THROUGH HS-SPME GC-MS:

(Logarithmic peak areas, mean $\pm$ standard deviation)

Table 2.13 volatile compounds present in kombucha determined through HS-SPME GC-MS

6 days	White tea	Black tea	Green tea	Chrysanthemum	Honeysuckle	Mint	ANOVA
ALCOHOLS							
phenyl ethyl alcohol	5.57 $\pm$ 0.02	5.43 $\pm$ 0.01	5.50 $\pm$ 0.12	5.89 $\pm$ 0.12	5.89 $\pm$ 0.12	5.50 $\pm$ 0.05	*
1-decanol	4.20 $\pm$ 0.12	4.36 $\pm$ 0.00	2.14 $\pm$ 3.03	4.36 $\pm$ 0.01	3.92 $\pm$ 0.03	4.03 $\pm$ 0.39	NS
Alpha-terpineol	3.96 $\pm$ 0.12	4.09 $\pm$ 0.09	4.24 $\pm$ 0.22	4.73 $\pm$ 0.00	3.80 $\pm$ 0.01	2.43 $\pm$ 3.44	NS
Linalool	4.33 $\pm$ 0.08	4.09 $\pm$ 0.09	4.24 $\pm$ 0.22	4.73 $\pm$ 0.00	3.80 $\pm$ 0.01	4.06 $\pm$ 0.25	***
1-hexadecanol	ND	ND	ND	ND	4.66 $\pm$ 0.23	ND	*
Pinocarveol	ND	ND	ND	ND	4.62 $\pm$ 0.07	ND	***
3-octanol	ND	ND	ND	ND	ND	5.23 $\pm$ 0.02	***
1-butanol, 3-	6.35 $\pm$ 0.10	6.44 $\pm$ 0.07	6.18 $\pm$ 0.16	6.43 $\pm$ 0.09	6.44 $\pm$ 0.03	6.33 $\pm$ 0.16	NS

methy-							
--------	--	--	--	--	--	--	--

6 days	White tea	Black tea	Green tea	chrysanthemum	Honeysuckle	Mint	ANOVA
ESTERS							
Decanoic acid, ethyl ester	3.99±0.06	3.93±0.61	2.18±3.08	4.09±0.31	1.93±2.73	2.44±3.45	NS
Hexadecanoic acid, ethyl ester	2.26±3.20	4.71±0.86	2.04±2.88	2.20±3.11	2.69±3.81	2.54±3.59	NS
Gamma-palmitolactone	2.10±2.97	3.82±0.13	1.81±2.57	2.03±2.88	1.97±2.79	3.58±0.03	NS
(-)-trans-dihydrocarvyl acetate	ND	ND	ND	ND	ND	2.36±3.34	NS

KETONES							
d-carvone	2.40±3.39	2.05±2.90	1.82±2.58	ND	4.13±0.29	6.19±0.19	**
dihydrocarvone	ND	ND	ND	ND	ND	5.43±0.19	**

jasmone	ND	ND	ND	ND	ND	4.45±0.35	NS
procymidone	ND	ND	ND	ND	4.14±0.13	ND	**

ACIDS							
Octonoic acid	6.07±0.03	6.27±0.12	6.07±0.21	6.07±0.02	6.08±0.13	6.20±0.18	NS
Deconoic acid	6.12±0.10	6.29±0.12	6.31±0.21	6.13±0.06	6.20±0.33	6.28±0.34	NS
Hexanoic acid	5.14±0.01	5.44±0.09	5.20±0.12	5.52±0.10	5.49±0.07	5.28±0.10	NS
ALDEHYDES							
2-decenal	4.17±0.18	3.58±0.04	1.89±2.68	ND	1.91±2.71	1.98±2.80	NS
Benzaldehyde	3.53±0.01	3.71±0.09	3.54±0.23	3.36±0.13	1.97±2.80	ND	NS

OTHERS							
--------	--	--	--	--	--	--	--

Indole	1.70±2.40	1.83±2.59	1.83±2.59	1.75±2.48	5.33±0.07	1.74±2.47	***
Eugenol	ND	ND	ND	4.54±0.17	ND	5.46±0.06	***
4-pyridone	ND	ND	ND	ND	6.05±0.02	ND	***

ND - Not detected, ANOVA: NS - Not significant.* =P<0.01. ***=P<0.001; different letters in same row mean samples are significantly different according to Tukey's HSD post-hoc test (P<0.05).

In Volatile composition of kombucha produced using each of the different substrates were evaluated at 6 days. At 0 hr and 12 days, the time point was used for the sensory analysis across all kombucha, 46 volatile compounds were detected including 15 alcohols, 9 esters, 7 fatty acids, 5 aldehyde, 5 ketones and 5 other compounds. In all kombucha, total of 22 compounds (mostly alcohols, acids and esters) were Aroma-active compounds were included that are called microbial metabolites such as ethyl alcohol (apricot aroma), p-ethylguaiacol (spice) and phenyl ethyl alcohol (lilac). Throughout the fermentation process the concentration of these compounds were increased. In honeysuckle kombucha, phenyl ethyl alcohol had higher degree of fermentation but was found in mint kombucha, which has lowest degree of fermentation (according to a tukey's HSD post-hoc test). In white, green, and black tea kombucha's few minor differences were observed in volatile profiles. The volatile compounds present in white tea kombucha were neo dihydrocarveol and p-methyl anisate, which were absent in the green and black tea kombucha.(Hara, 1995)

In some herbal kombucha another 17 compounds were present but were not present in the tea-based kombucha. In mint and chrysanthemum kombucha, Eugenol- a sweet, woody scent was found. Many carveol alcohols (trans-carveol, sis-carveol, and pinocarveol) and p-cymenene were found in the honeysuckle and mint kombucha. Volatile compounds such as dihydro-carveol, 3-octanol, α -cardinol, (-) - trans-dihydrocaryl acetate, dihydro-carvone, piperitenone and jasmone were observed in mint kombucha. These compounds are typical to mint, contributing to aroma of refreshing scent(Kaneko *et al.*, 2017; Padmini *et al.*, 2010). In could be concluded that all the kombuchas prepared in the present study retained the characteristics of aroma

compounds of their substrates during the fermentation process, evaluating kombucha aroma. To analyze the variety profile PCA was performed. The PCA biplot reveals bunch of compounds associated with either mint, tea-based kombucha had relatively similar volatile compounds clustered together along with the chrysanthemum kombucha. Eugenol was the only detected volatile compound from chrysanthemum sample that are distinguished from tea-based kombucha, and their fermentation dynamics were most similar to those of the tea-based kombucha. (Zhang *et al.*, 2021)

CHAPTER 3

AIM AND OBJECTIVES

AIM:

To prepare the fermented kombucha drink with an infused oyster mushroom extract.

OBJECTIVES:

- 1.To cultivate the oyster mushroom and extract the powder.
- 2.To prepare the fermented drink kombucha.
- 3.To analyze the drink for its phenolic content and antioxidant activity.
- 4.To analyze the antimicrobial activity of kombucha.

CHAPTER 4

MATERIALS AND METHODS

4.1 CULTIVATION OF OYSTER MUSHROOM

For the preparation of kombucha tea, oyster mushrooms were cultivated and grown at room temperature. *Pleurotus ostreatus*, also known as oyster mushroom which is an edible mushroom, was cultivated for the fermentation of kombucha. To cultivate oyster mushroom we need substrate such as paddy straw, mushroom spawn, coir pith, coffee powder (waste), sugar cane waste, polythene bag (60*30) cm was used as a substrate to grow oyster mushrooms. For the preparation of the bag, first a paddy straw was collected and soaked in water for a few hours. After that it was dried for a few minutes and packed in a bag and closed the opening of the bag tightly. The packed paddy straw was put on autoclave for 15 minutes at the temperature of 121°C, pressure at 15 psi (pounds per square inch). Then it was left for 20 minutes to kill the bacteria present in it. Autoclave was done for the sterilization purpose. After the autoclave was over, it was dried in a sterilized area to reduce the heat (vaporized condition). Then, coir pith was soaked in water for a few hours (2 hours). The size of coir pith increased when it was soaked in water. Then the coir pith was taken and left them for a few minutes to dry. After drying it was taken and packed in a bag. After packing them in a bag it was kept in an autoclave to sterilize it. Coir pith were autoclaved for 15 minutes at 121°C of temperature, for 15 psi pressure. After the autoclave was over, it was dried in a sterilized area to reduce the heat.

After that, for packing the bag, the coir pith and coffee powder was measured in an weighing machine for the bag packing. After measuring the required amount of coir pith and coffee powder, packing of bag was done. Then, the coir pith and coffee powder were mixed together after measuring them to put it in the bag. For packing the bag, few steps were involved for packing process;

- 1) Polythene bag was taken of about the size of (60*30) cm. Then the bottom of the bag was closed tightly.
- 2) Then the paddy straw was taken and it was rolled like a spring shape or like a coil in shape tightly and it was arranged in a polythene bag as layers.
- 3) Then the coir pith and coffee powder which were measured and mixed together was taken and spreader around the corners of the bag above the paddy straw as the second layers.
- 4) After that handful of mushroom spawns (oyster mushroom) was taken and it was scattered the layers of coir pith and coffee powder gently as the third layer.
- 5) The steps were repeated again until it reaches 5-6 layers.
- 6) After it reached the maximum layers the polythene bag was closed tightly at the top of the bag.

After the bag packing was done, the bag was hung under the hanger with some distance. The bag was observed daily for the growth of mushrooms. It takes a maximum of 25 days to 1 month to grow. The bag was observed regularly and the water was sprayed daily for moisture. Between 15-20 days, the mycelium growth was seen. After 20 days mycelium was spread around the bag and the bud formation was seen. After the bud was grown at maximum size it was grown at maximum size it was harvested and preserved them at the freezer at -21°C. Nearly 8 to 10 bags were packed for the growth of mushroom by following same procedure and steps for the purpose of project use. Mushroom bag was observed daily and growth was monitored daily from day 1 to day 25 as follows:

Day 1; Bag was packed by using required substrate and was hung on the hanger to grow. Filter paper was placed with some distance under the bag for moisture. Small holes were put on the bag for the growth of buds. Then, water was sprayed on the bag and on the filter paper and was kept in a sterilized area to avoid contamination.

Day 2; Bag was observed. No growth was seen, water was sprayed on the bag and on the filter paper and left them to grow without disturbing.

Day 3; Observation of bag was done. Water was sprayed. No growth was seen.

Day 4; Water was sprayed on the bag and no growth was seen.

Day 5; Bag was observed and water was sprayed. Mycelium was observed in a moisture content as sprouts.

Day 6; Water was sprayed around the mycelium to maintain its moisture content.

Day 7; Mycelium growth was seen. Water was sprayed throughout the bag.

Day 8; Water was sprayed and mycelium growth was observed.

Day 9; Mycelium spreads all over the bag. Water was sprayed around the bag.

Day 10; Formation of mycelium was observed. Mycelium was observed as root like structure and spread all over the bag. Water was sprayed around the bag.

Day 11; Mycelium spreads throughout the bag. Water was sprayed to maintain moisture content.

Day 12; Mycelium growth was observed and mycelium spread all over the bag. Its density increases day by day and water was sprayed to prevent dryness.

Day 13; Mycelium density increases. Water was sprayed to maintain moisture content.

Day 14; Water was sprayed throughout the bag and mycelium growth was observed.

Day 15; Mycelium density increases were observed. Water was sprayed to keep them moist.

Day 16; Growth of mycelium increases day by day. Water was sprayed to maintain moisture content for the growth of mycelium.

Day 17; Mycelium growth increases and to maintain moisture content water was sprayed throughout the bag.

Day 18; Mycelium starts developing into buds. Water was sprayed all over the bag.

Day 19; Slightly bud formation was seen. Water was sprayed to maintain its moisture content.

Day 20; Bud grows bigger in size and water was sprayed to prevent dryness.

Day 21; The bud develops into a fruiting body and water was sprayed throughout the bag.

Day 22; The fruiting body develops to attain its maximum growth. Water was sprayed to maintain moisture content.

Day 23; Fruiting body grows bigger and reaches its maximum growth and develops into an oyster mushroom.

Day 24; Fruiting body grows bigger and reaches its maximum growth and develops into an oyster mushroom.

Day 25; First mushroom was harvested by using a sterile knife to avoid contamination and preserved in the freezer at -121°C.

As the day passed, mycelium grew bigger and bigger, Bud formation was seen and the bud developed into a fruiting body then the fruiting body develops into an oyster mushroom. These are the steps and procedures involved for the cultivation and growth of the oyster mushroom.

4.2 MUSHROOM POWDER EXTRACTION

4.2.1 MATERIALS USED

Oyster mushroom, Mortar and pestle, Beaker, Ethanol, Distilled water.

4.2.2 METHODOLOGY

4.2.2.1 Oyster mushroom powder

Freshly grown oyster mushrooms were harvested and then the mushroom was freeze dried and cut into pieces. Then, small pieces of mushroom were crushed into powder form with the help of a mortar pestle.

4.3 PREPARATION OF KOMBUCHA

To start with the preparation of kombucha, a sterilized vessel was taken. Then, two liters of water was measured. After that water was brought to boil. Water was allowed to boil for 5-10 minutes. After boiling of water, 15 grams of tea and 45 grams of sugar were added. Altogether, it was boiled until the flavor of tea was completely flavored in water. After the flavor of tea was enhanced in water, turn off the stove and the tea were allowed to cool down to room temperature. A clean fermenting glass jar was taken for the fermentation process of kombucha. Glass jar was taken and washed

well with hot water. Then, it was wrapped with newspaper and kept in an autoclave for sterilization. After the autoclave was done, a glass jar was taken and allowed to cool down to room temperature. After the two glass jars and tea reached the normal room temperature, an equal amount of ready-made raw material kombucha was poured into two jars. Then, the equal amount of SCOBY was added to both the jars. One jar is for testing, and another jar is for control. After that the black tea was measured equally and added to both the jars. In the control jar, the added substrate was ready-made raw kombucha, SCOBY and Black tea and on the other hand the added substrates in the test jar were ready-made kombucha, SCOBY, black tea and Oyster mushroom powder. The process and preparation of kombucha methods was over. Both the jars were kept in dark, warm places, close the jars tightly. Jars were kept in warm places without disturbing them. After that it goes for the fermentation process. Fermented kombucha takes 14 days to ferment.

4.4 COLLECTION OF SAMPLES

For the collection of kombucha samples, sterile vials were taken. Sample was collected on both the test and control jar. Then, the kombucha drink sample was collected on the 0th day (initial day) followed by the 7th day and 14th day. On these three days, samples were collected and stored in sterile vials to study the properties of kombucha, the components present in kombucha, antioxidant properties, anti-inflammatory properties etc., samples were taken for the analysis purpose. To analyze the sample, GC/MS (Gas chromatography/ Mass spectrometry) method was used to identify the components present in kombucha. Then, on the 14th day samples were taken and tasted. Further, sensory analysis was performed to find out the overall flavor, taste and texture of kombucha. After tasting kombucha tea, some feel a pleasant fruity and sour taste, vinegar taste while others feel pleasant fruity and sour.

4.5 ANTIMICROBIAL TEST:

4.5.1 MATERIALS REQUIRED:

The materials used are

- Muller Hinton Agar
- petri plates
- Bacterial broth culture swabs

- Discs / well cutter
- Forceps

4.5.2 PROCEDURE:

This method was done by preparing Muller Hinton Agar plates and swabbing the organisms such as *E. coli*, *Salmonella*, *S. aureus*, *Pseudomonas* and *Vibrio cholerae* to check the antibacterial property of kombucha tea. The procedure as follows:

1. Firstly, the petri plates and the media were sterilized in an autoclave at 121°C for 15 mins.
2. Then, the plates were taken to the laminar air flow chamber along with the media. Then the media is poured into the petri plates and allowed to solidify.
3. These petri plates were then swabbed with the *Salmonella* and *E. coli* using sterile swabs.
4. After swabbing wells are cut into the plates using sterile pipette tips. And then the wells were loaded with the kombucha samples. Four wells for the concentration of 10µl, 25µl, 50µl and 100µl were loaded respectively.
5. The plates were observed the next day for the presence of inhibition circles or the clear zone around the wells.

4.6 pH test:

The pH level was tested for 0th day, 7th day and 14th day samples using pH paper and pH meter. The kombucha drinks were taken in sterile beakers and measured in pH meter.

4.7 ANTIOXIDANT TEST:

The DPPH assay (2,2-diphenyl-1-picrylhydrazyl) is a commonly used method for measuring the antioxidant activity of the compounds. This DPPH assay involves the use of a stable free radical, DPPH which changes the color from purple to yellow when reduced by an antioxidant compound. The scavenging activity of the antioxidant indicates the degree of the color change or reduction in absorbance. The higher the degree of color change the higher the antioxidant activity.

4.7.1 MATERIALS REQUIRED: -

1. 96 well plate
2. Pipette
3. 0.0024 of DPPH-alpha diphenyl
4. Beta-picrylhydrazyl
5. Ethanol
6. Methanol
7. DMSO-dimethyl sulfoxide
8. Kombucha samples
9. UV spectrophotometer

4.7.2 PROCEDURE:

1. As DPPH is photosensitive, it should be performed in a dark room.
2. To perform the DPPH assay, the DPPH solution is prepared in a solvent such as ethanol and methanol 0.0024g of DPPH is added to 50ml of methanol and mixed it well
3. The different concentrations of kombucha samples such as 0C, 0T, 7C, 7T, 14C, 14T were added in the well plate. Concentrations such as 25µl, 50µl, 75µl, 100µl of kombucha samples were added. A duplicate method is used for reducing the error while performing the test.
4. The samples are diluted with different concentrations of distilled water by adding it in reverse to the concentration of samples.
5. Then, 200µl of DPPH was added to the well containing samples after diluting the samples.
6. Blank and control wells are prepared on the last 2 rows of the plate.
7. Then, the well plate was incubated for 1hr at room temperature in a dark room.
8. After incubation, the color of the sample's changes from violet to yellow.
9. Then run the plate in a UV spectrophotometer at the wavelength of 515 nm.
10. Then, the readings are interpreted & calculated and the results are represented as a graph.

4.8 PHENOLICS TEST:

Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity. The total phenolic content of the extract was determined by folin-Ciocalteu assay, also known as the FC assay.

4.8.1 MATERIALS USED:

- 96 well plate
- Sodium carbonate
- Folin-Ciocalteu reagent
- Gallic acid
- Kombucha samples
- UV spectrophotometer
- Pipettes

4.8.2 METHODS:

1. To perform phenolics test or FC assay, FCP reagent is prepared in 1:10 ratio i.e., 1 ml of Folin-ciocalteu reagent is mixed in 9 ml of distilled water.
2. Then, different concentration of samples such as 0C, 0T, 7C, 7T, 14C, 14T were added in the 96 well plate at the concentration of 40 μ l, 30 μ l, 20 μ l, 10 μ l. Duplicate method is used for reducing the errors while performing the test.
3. The sample are diluted with different concentration of distilled water by adding it in reverse to the concentration of samples.
4. After the sample gets diluted, 100 μ l of Folin-ciocalteu reagent is added to the samples and kept aside for 1 min.
5. Then, add 60 μ l of sodium carbonate to the sample slowly.
6. After that different concentration of gallic acid were added as same as in the sample and dilute it with distilled water. Gallic acid was added to find the control for the test.
7. Then add 100 μ l of Folin-ciocalteu reagent and 60 μ l of sodium carbonate to the gallic acid.
8. In gallic acid and in the samples added in the well plate, bubble was observed while adding sodium carbonate.

9. After that the plate was incubated for 1 hour at room temperature. After the incubation period was over, run the plate in a UV spectrophotometer at a wavelength of 750 nm.
10. Then readings are calculated and the results are represented as a graph.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 MUSHROOM CULTIVATION:

The properties of nutritional and medicinal content are well known in Oyster mushrooms. They were used for various purposes such as food, producing drug, and to treat some disease. The mushroom bud formation is done after 24-26 days from spawning. Several buds are formed and cultivated them according to their growth.

5.2 YIELD:

The yield of the first bud mushroom was 270 grams, and the next bud was 340 grams. Yield of our first bag is 610 grams (0.61) kg.



Fig 5.2.1 mushrooms grown on bags

5.3 MUSHROOM EXTRACTION:

The oyster mushroom extract preparation process done and the powdery extract is given below.

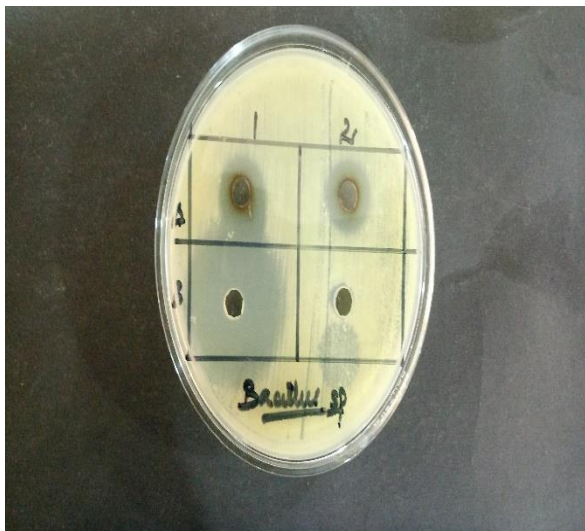


Fig 5.3.1 Mushroom extract powder

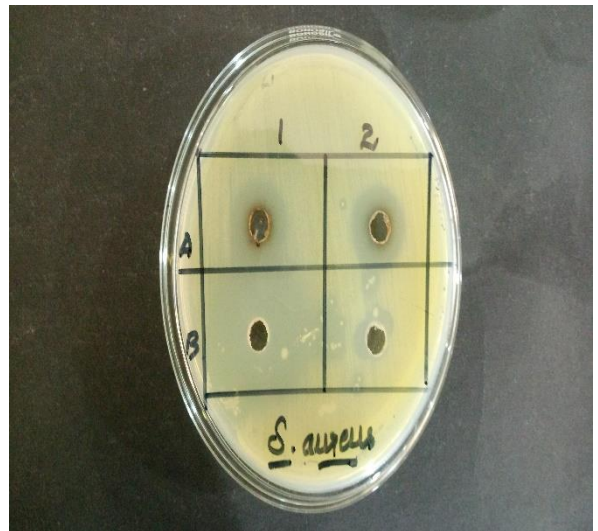
5.4 ANTI-MICROBIAL TEST:

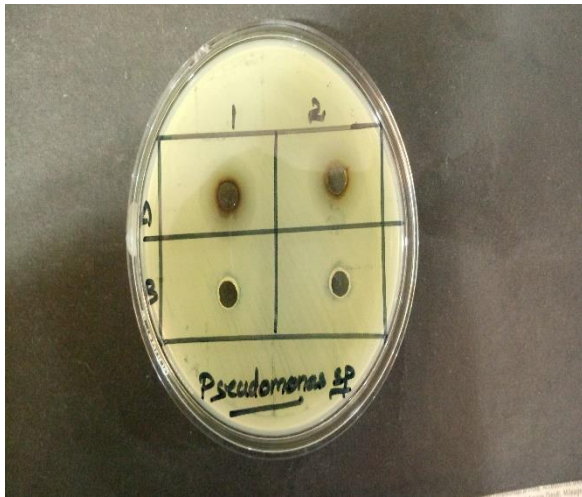
For each well, zone of inhibition was observed after 24 hours and measured and recorded as table shown below.

Bacillus sp.

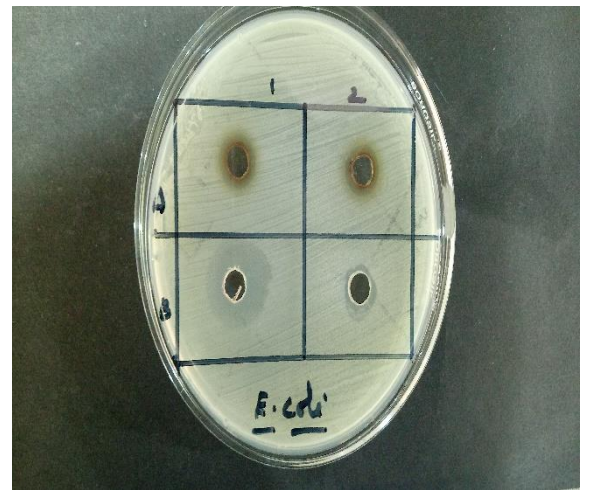


Staphylococcus aureus





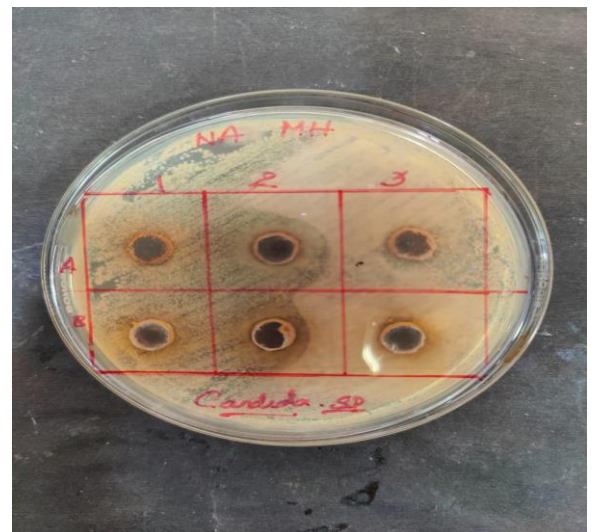
Pseudomonas sp.



E. coli



Aspergillus sp.



Candida albicans

Fig 5.4.1 Plate of anti-microbial test showing zone of inhibition

Table 5.4.1 Results for antimicrobial test done with mushroom infused kombucha samples against traditional kombucha.

SAMPLES	Zone of inhibition in mm in diameter Test organisms					
	<i>S. aureus</i>	<i>Pseudomonas sp.</i>	<i>E. coli</i>	<i>Bacillus sp.</i>	<i>C. albicans</i>	<i>Aspergillus sp.</i>
A1	S (10 mm)	S (8 mm)	S (7 mm)	S (8 mm)	R	S (8 mm)
B1	S (28 mm)	R	S (12 mm)	S (26 mm)	R	R
A2	S (8 mm)	S (8 mm)	R	S (8 mm)	S (8 mm)	R
B2	S (6 mm)	R	RC	R	S (7 mm)	R

- A1, A2, shows the control (traditional kombucha) and B1, B2, shows the test (mushroom infused kombucha) of 0th day and 14th day samples respectively.

Results showed that the zone of inhibition is formed in species such as *S. aureus*, *Pseudomonas sp.*, *E. coli*, *Bacillus sp.*, *Aspergillus sp.* and *C. albicans* significantly. These results were similar to that of the work done by (fateme valiyan *et al.*) These results interpret significant antimicrobial activity of mushroom infused kombucha tea as compared to traditional kombucha tea. These antimicrobial activity of kombucha tea is due to the lactic acid present in it which is produced by the *Lactic Acid Bacteria* (LAB), *Lactobacillus* and other species.

5.5 pH TEST:

The readings taken from the pH meter showed the following pH levels for 0th day control 4.82, 0th day test 4.96, 7th day control 4.93, 7th day test 5, 14th day control 6.17 and 14th day test. The pH was acetic at the start of the fermentation i.e., at 0th day and 0th gradually decreased with increase in fermentation time. On 14th day, the pH level came to slightly acidic which is in consumable range. The readings from pH meter are shown in the figures below:

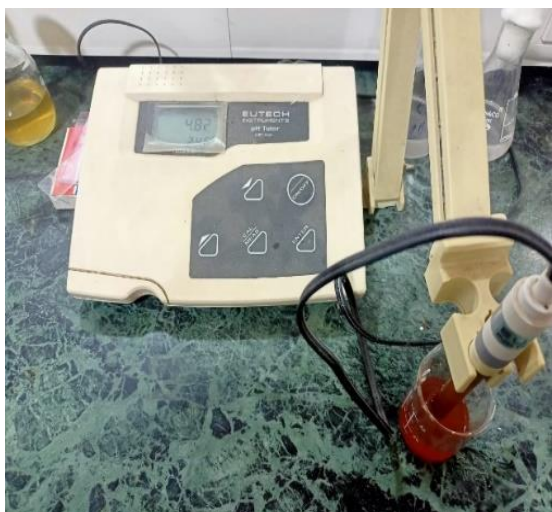


Fig 5.5.1 pH reading of 0 C

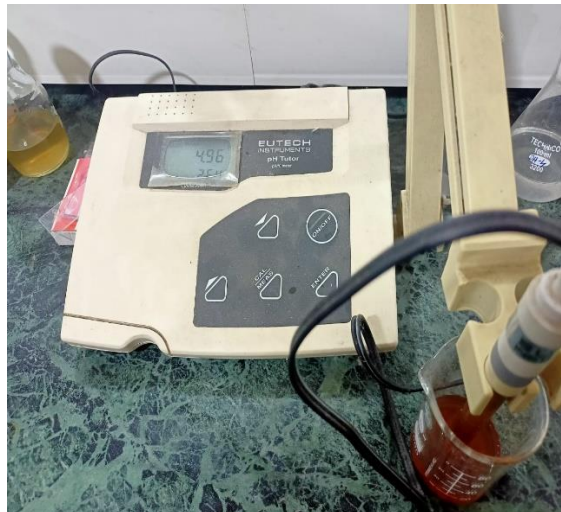


Fig 5.5.2 pH reading of 0 T



Fig 5.5.3 pH reading of 7 C



Fig 5.5.4 pH reading of 7 T



Fig 5.5.5 pH reading of 14 C



Fig 5.5.6 pH reading of 14 T

5.6 ANTI-OXIDANT TEST:

This assay is based on the measurement of the scavenging capacity of antioxidants towards it. The 96-well plates with samples are run in an UV spectrometer at 515 nm. Results (color change) were observed after 1 hour, the G & H row contains the control and tabulated in table 5.6.1

Table 5.6.1 table showing readings from spectrophotometry

CONCENTRATION		0TH DAY		7TH DAY		14TH DAY	
		OC	OT	7C	7T	14C	14T
25		0.666	0.58	0.956	0.929	1.147	0.839
25		0.754	0.864	0.976	0.87	0.476	0.767
50		0.575	0.814	0.661	0.763	0.465	0.511
50		0.679	0.067	0.779	0.852	0.486	0.653
75		0.596	0.786	0.553	0.816	0.344	0.52
75		0.533	0.787	0.71	0.682	0.413	0.452
100		0.435	0.82	0.637	0.768	0.298	0.445
100		0.447	0.66	0.692	0.55	0.322	0.516

The percentage of the RSA (Radical Scavenging Activity) is calculated using the formula,

$$\text{Scavenging (\%)} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the treated sample})}{\text{Absorbance of the control}} \times 100$$

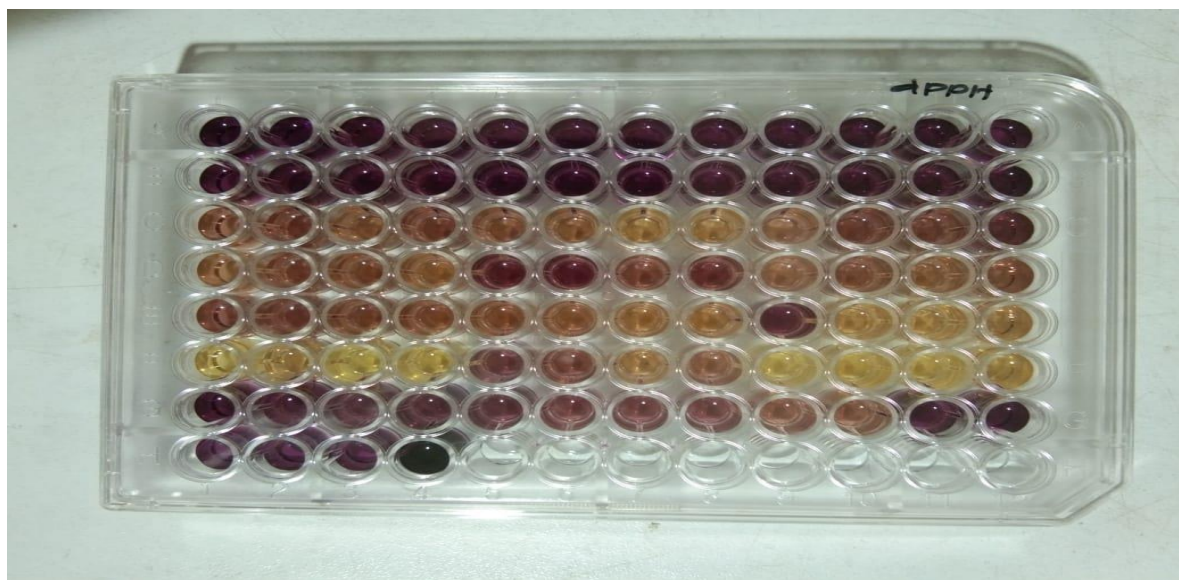


Fig 5.6.1 DPPH assay on 96- well plate

Table 5.6.2 table showing average value of DPPH assay readings from spectrometry.

CONCENTRATION	0TH DAY		7TH DAY		14TH DAY	
	OC	OT	7C	7T	14C	14T
25	0.71	0.786	0.966	0.899	0.811	0.803
50	0.627	0.74	0.72	0.807	0.475	0.582
75	0.564	0.722	0.664	0.549	0.378	0.486
100	0.441	0.44	0.631	0.659	0.31	0.48

The control used in the test is ascorbic acid.

Control 1 - 0.417 OD

Control 2 - 0.408 OD

Control average - 0.4125 OD

*OD- Optical Density

Table 5.6.3 table showing final RSA % of samples

CONCENTRATION	0TH DAY		7TH DAY		14TH DAY	
	OC	OT	7C	7T	14C	14T
25	41.9	47.51	57.29	54.11	49.13	48.63
50	34.21	44.25	42.7	45.1	24.84	29.12
75	26.86	42.86	37.87	37.4	13.15	15.12
100	6.42	6.25	34.62	31.83	8.36	14.06

The data presented in table 5.6.3 shows that antioxidant activity of traditional kombucha (control) and *P. ostreatus* infused kombucha (test) were increased with fermentation time to reach the highest values after 0th day, 7th day and 14th day of fermentation time of kombucha respectively at different concentrations which were measured and readings were taken in spectrophotometry. The DPPH (RSA%) scavenging capabilities decreases with increase in concentration level. The concentration level of 25 µL, 50µL, 75µL and 100µL were measured for each sample. The OD readings of 0th day control sample are observed and shown the following trend 41.9 >34.21 >26.86 >6.42 for respective concentrations. The OD readings of 0th day test samples were observed and shown the following trend 47.51>44.25>42.86>6.25 for respective concentrations. The OD readings were measured and observed for 7th day control sample and shown the following trend 57.29>42.7>37.87>34.62 for respective concentrations. The OD readings were measured and observed for the 7th day test and showed the following trend 54.11>45.1>37.4>31.83 for respective concentrations. The OD readings were measured and observed for 14th day control sample and showed the following trend 49.13>24.84>13.15>8.36 for respective concentrations. The OD readings were measured and observed for 14th day test sample and shown the following trend 48.63>29.12>15.12>14.06 for respective concentrations. The above data concludes that at 25 µL concentration, the maximum antioxidant activity was observed and at 100 µL concentration, the minimum antioxidant activity was observed. The highest antioxidant activity was seen in 7th day control (57.29 OD) and the lowest antioxidant activity was seen in 0th day control (6.42 OD). According to (amarasinghe *et al.*,2018 and lobo *et al.*,2017), the antioxidant activity increases with increase in the fermentation time of kombucha. Compared to the 0th day sample, the 7th day sample shows the highest antioxidant activity with increase in fermentation time of kombucha.

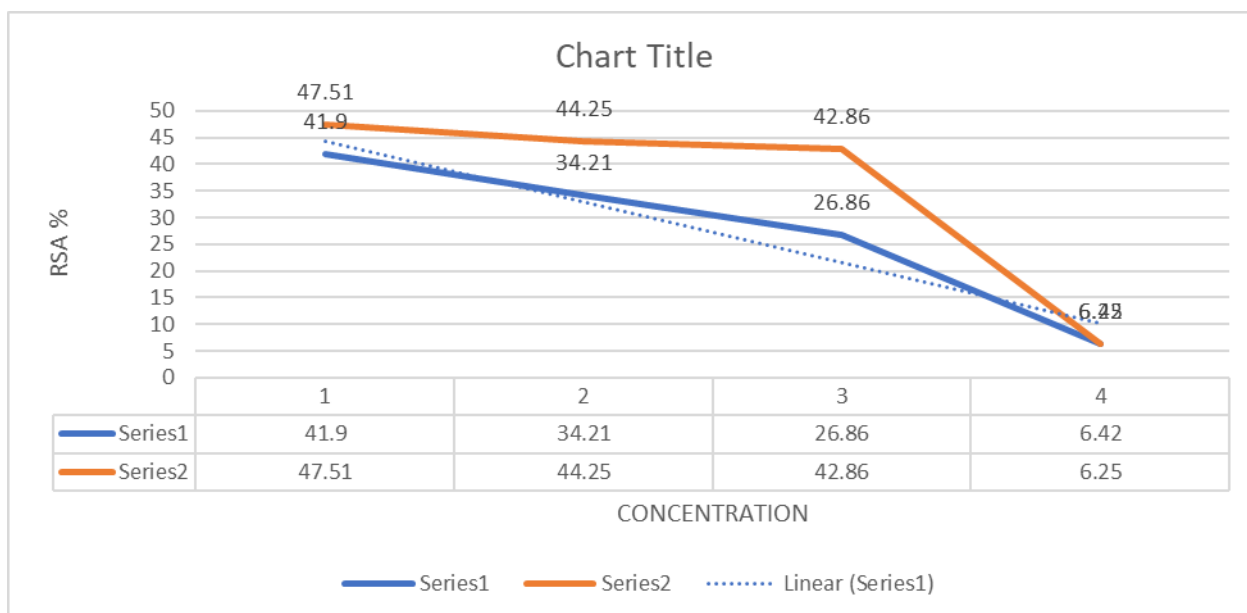


Fig 5.6.2 graph showing antioxidant activity of kombucha on 0th day.

In the above graph, the series 1 represents 0th day control and the series 2 represents 0th day test. The X axis represents concentration of kombucha at 25 μ L, 50 μ L, 75 μ L, 100 μ L at 1,2,3,4 respectively and the Y axis represents the RSA%.

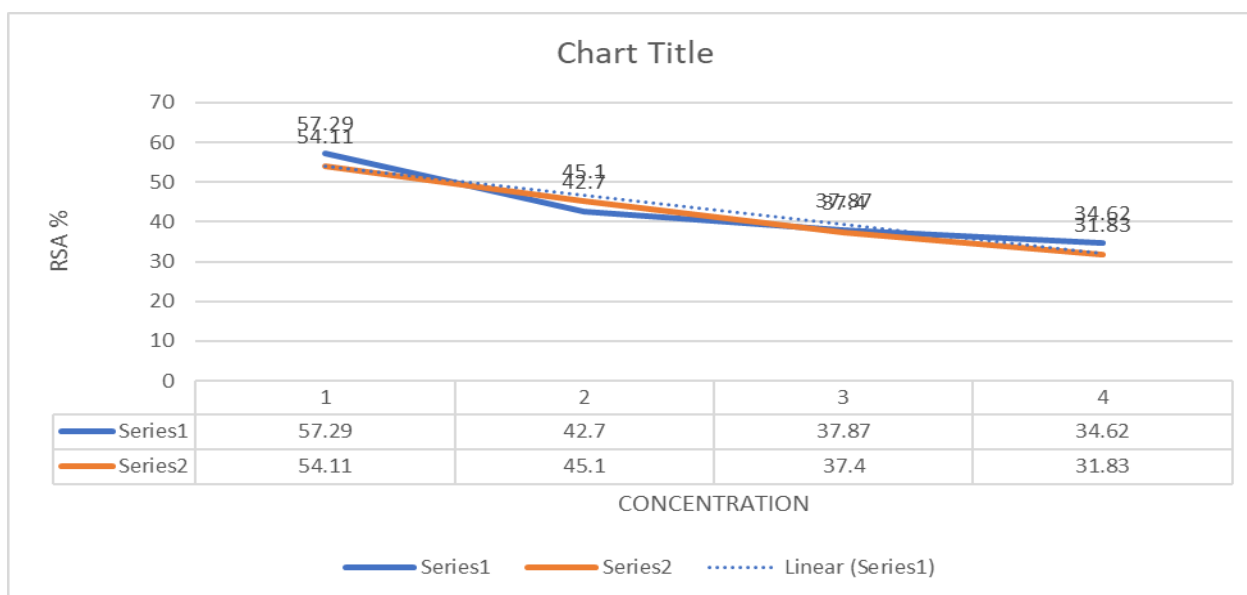


Fig 5.6.3 graph showing antioxidant activity of kombucha on 7th day

In the above graph, the series 1 represents 7th day control and the series 2 represents 7th day test. The X axis represents concentration of kombucha at 25 μ L, 50 μ L, 75 μ L, 100 μ L at 1,2,3,4 respectively and the Y axis represents the RSA%.

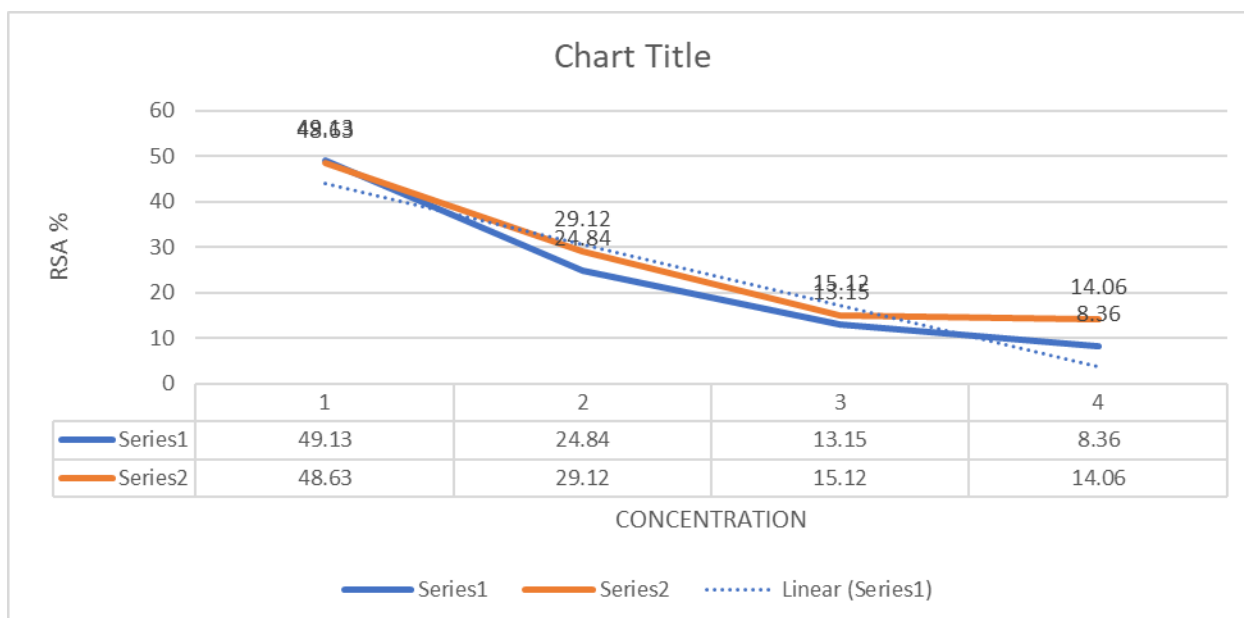


Fig 5.6.4 Graph showing antioxidant activity of kombucha on 14th day

In the above graph, the series 1 represents 14th day control and the series 2 represents 14th day test. The X axis represents concentration of kombucha at 25 μ L, 50 μ L, 75 μ L, 100 μ L at 1,2,3,4 respectively and the Y axis represents the RSA%.

5.7 PHENOLICS TEST

The phenols test by folin-ciocalteu reagent proves the reduction of folin-ciocalteu reagent in the presence of phenols that is spectrophotometrically at 750 nm. The reading is shown in the tables listed below:

Table 5.7.1 table showing the readings of phenolic test

CONCENTRATION	0TH DAY		7TH DAY		14TH DAY	
	OC	OT	7C	7T	14C	14T
25	0.336	0.14	0.304	0.311	0.424	0.571
25	0.338	0.279	0.301	0.549	0.665	0.609
50	0.433	0.152	0.381	0.673	0.74	0.843
50	0.423	0.505	0.392	0.538	0.639	0.498
75	0.499	0.634	0.449	0.559	0.667	0.691
75	0.471	0.541	0.336	0.833	0.869	0.624
100	0.54	0.663	0.421	1.034	0.075	0.765
100	0.594	0.728	0.525	0.969	0.009	0.906

To calculate the RSA% we need to find the average values of the sample in different concentrations and the control,

$$\text{Scavenging (\%)} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the treated sample})}{\text{Absorbance of the control}} \times 100$$

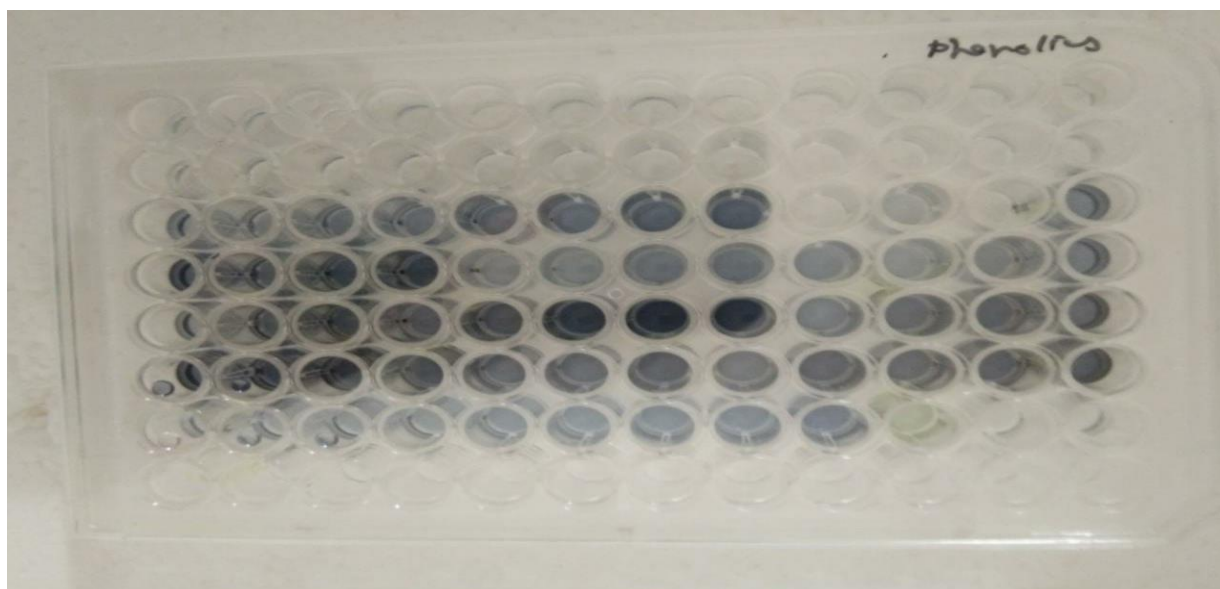


Fig 5.7.1 phenolics test on 96-well plate

Table 5.7.2 table showing average value of phenolic test

CONCENTRATION	0TH DAY		7TH DAY		14TH DAY	
	OC	OT	7C	7T	14C	14T
25	0.337	0.209	0.302	0.43	0.544	0.59
50	0.428	0.328	0.386	0.605	0.689	0.67
75	0.485	0.587	0.392	0.696	0.768	0.657
100	0.567	0.695	0.473	1	1.04	0.835

The control used in the test is gallic acid.

Control 1 - 0.196 OD

Control 2 - 0.205 OD

Control average - 0.2005 OD

OD - Optical Density

Table 5.7.3 table showing RSA% of phenolics test

CONCENTRATION	0TH DAY		7TH DAY		14TH DAY	
	0C	0T	7C	7T	14C	14T
25	36.89	4.06	33.6	53.37	63.14	66.01
50	52.1	38.87	48.05	66.85	70.89	69.48
75	58.65	65.84	48.85	71.19	73.89	70.07
100	64.63	71.15	57.61	79.95	80.72	75.98

The data presented in table 5.7.3 shown that the phenolic content of traditional kombucha (control) and the *P. ostreatus* infused kombucha (test) were increased with fermentation time to reach the highest value of fermentation time of 0th day, 7th day, 14th day of kombucha respectively at different concentration which were measured and reading in taken in spectrophotometer. The total amount of phenolic content in kombucha increases with increase in concentration level. The concentration level of 25 µL, 50µL, 75µL and 100µL were measured for each sample. The OD readings of the samples of the 0th day control is observed and shown the following trend 36.89< 52.1<58.65<64.63 for respective concentration. The OD readings of the samples of the 0th day test is observed and shown the following trend 4.06<38.87<65.84<71.15 for respective concentration. The OD readings of the samples of the 7th day control is observed and shown the following trend 33.6<48.05<48.85<57.61 for respective concentration. The OD readings of the samples of the 7th day test is observed and shown the following trend 53.37< 66.85<71.19<79.95 for respective concentration. The OD readings of the samples of the 14th day control is observed and shown the following trend 63.14< 70.89<73.89<80.72 for respective concentration. The OD readings of the samples of the 14th day test is observed and shown the following trend 66.01<69.48<70.07<75.98 for respective concentration. It was found that the phenolic compound was increased gradually with respect to fermentation time, phenolic compounds are regarded as high-level antioxidants because of their ability to scavenge free radicle and active oxygen species. The above data concludes that at 25 µL concentration the minimum phenolic content was seen and at 100 µL concentration the maximum phenolic content was observed. The highest phenolic content was observed in 14th day control (80.72 OD) and the lowest phenolic content

was observed in 0th day test (4.06 OD). This shows that according to (Vijayaraghava *et al.*,2000 and srihari, Satyanarayana *et al.*,2012) stated that the phenolic compounds were increased with increase in fermentation time of kombucha. Compared to the 0th day sample and fermentation time of kombucha, 14th day sample shows highest phenolic content in fermentation time of kombucha.

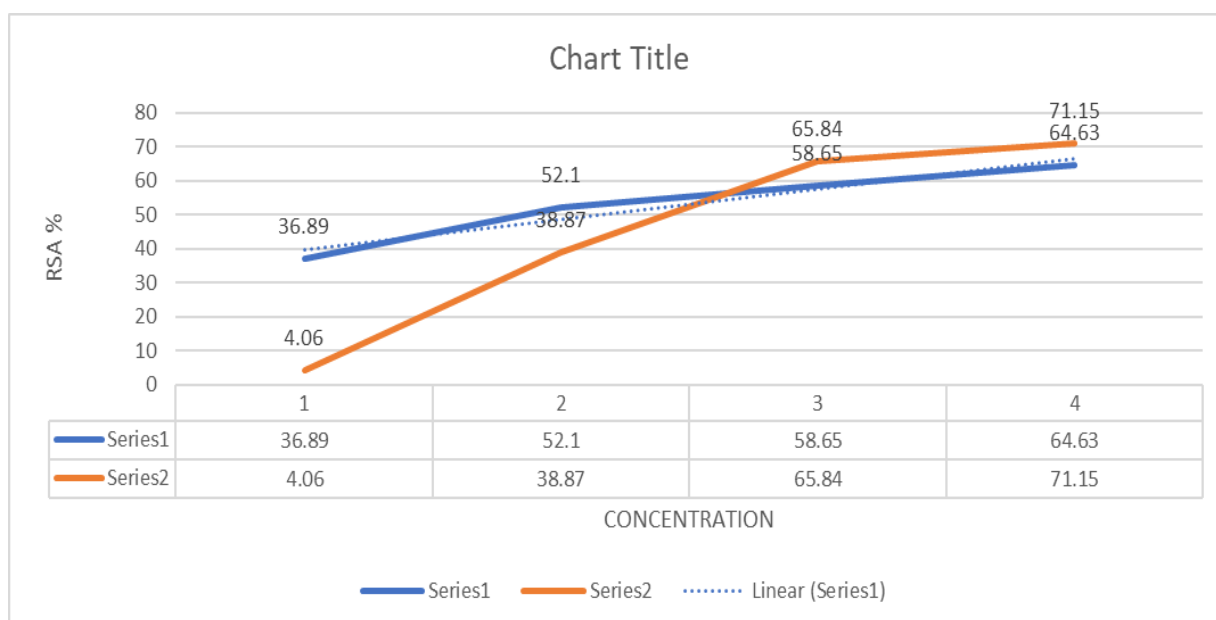


Fig 5.7.2 graph showing the total phenolic content of kombucha on 0th day

In the above graph, the series 1 represents 0th day control and the series 2 represents 0th day test. The X axis represents concentration of kombucha at 25 μ L, 50 μ L, 75 μ L, 100 μ L at 1,2,3,4 respectively and the Y axis represents the RSA%.

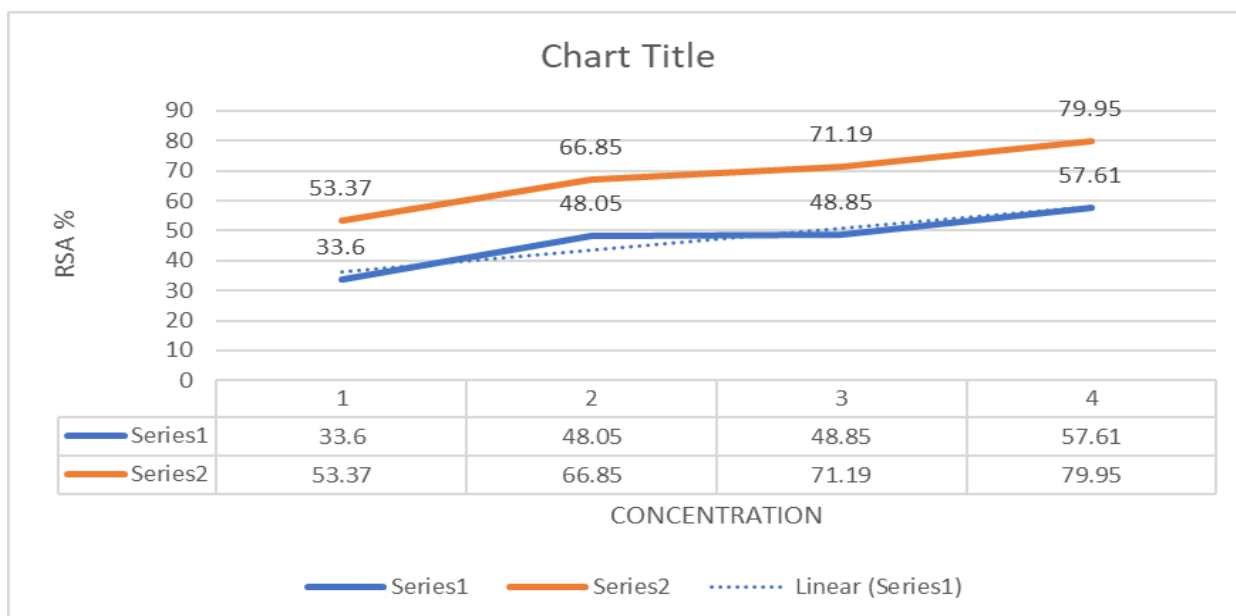


Fig 5.7.3 graph showing the total phenolic content of kombucha on 7th day

In the above graph, the series 1 represents 7th day control and the series 2 represents 7th day test. The X axis represents concentration of kombucha at 25 μ L, 50 μ L, 75 μ L, 100 μ L at 1,2,3,4 respectively and the Y axis represents the RSA%.

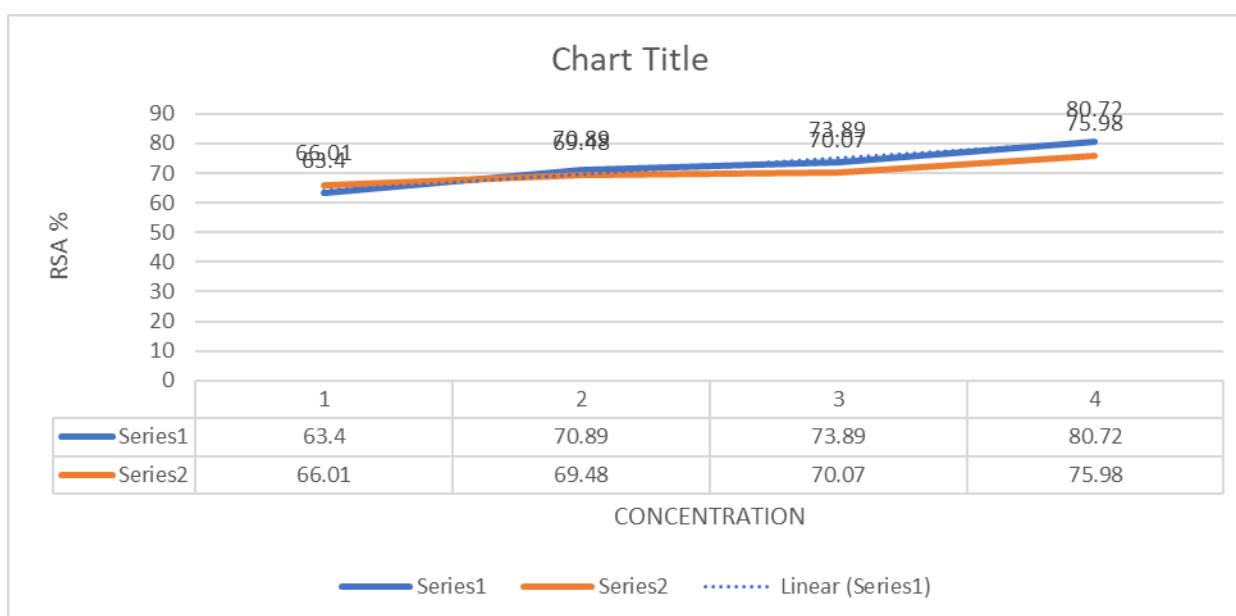


Fig 5.7.4 graph showing the total phenolic content of kombucha on 14th day

In the above graph, the series 1 represents 14th day control and the series 2 represents 14th day test. The X axis represents concentration of kombucha at 25 μ L, 50 μ L, 75 μ L, 100 μ L at 1,2,3,4 respectively and the Y axis represents the RSA%.

CHAPTER 5

SUMMARY AND CONCLUSION

Kombucha is a popular fermented drink among many traditional fermented beverages which is originated in China. Kombucha has many useful properties studied under in vivo, invitro environment such as antioxidants, anti-inflammatory, antibacterial, antidiabetic, anticarcinogenic activity. The results from our analysis demonstrates that the kombucha has high antioxidant activity which works on several diseases like diabetes and cancer. According to (*Amarasinghe et al.,2018 and lobo et al.,2017*) the antioxidant activity increases with increase in the fermentation time of kombucha. The results shows that when compared to 0th day sample, the 7th day sample showed high antioxidant activity with increase in fermentation time. Total phenolic content was measured by using Folin-ciocalteu reagent according to (*Singleton and rossi et al.,1965*). The result presented in our study showed that the total phenolic content in kombucha was higher in 14th day sample compared to 0th day sample. According to (*Vijayaraghava et al.,2000 and srihari and satyanarayana et al.,2012*) reported that the total phenolic content in the kombucha samples increases with increase in the fermentation time of kombucha. Above results from our study concluded that compared to 0th day sample, the 14th day sample shows highest phenolic content in kombucha with increase in fermentation time. Fermentation time and sugar concentration showed a significant effect on antimicrobial activity of kombucha beverages. The most antimicrobial activity was observed against *Staphylococcus aureus*. The pH range of kombucha drink ranges from 4-7 which is similar to that of (*Chakravorty et al.,2009*).

References:

1. Avci, E., Avci, G. A., & Kose, D. A. (2014). Determination of antioxidant and antimicrobial activities of medically important mushrooms using different solvents and chemical composition via GC/MS analyses. *Journal of Food and Nutrition Research*, 2(8), 429–434.
2. Barros, L., Correia, D. M., Ferreira, I. C. F. R., Baptista, P., & Santos-Buelga, C. (2008). Optimization of the determination of tocopherols in *Agaricus* sp. edible mushrooms by a normal phase liquid chromatographic method. *Food Chemistry*, 110(4), 1046–1050.
3. Batista, P., Penas, M. R., Pintado, M., & Oliveira-Silva, P. (2022). Kombucha: Perceptions and Future Prospects. *Foods*, 11(13), 1977.
4. Battikh, H., Bakhrouf, A., & Ammar, E. (2012). Antimicrobial effect of Kombucha analogues. *LWT-Food Science and Technology*, 47(1), 71–77.
5. Bhattacharya, D., Bhattacharya, S., Patra, M. M., Chakravorty, S., Sarkar, S., Chakraborty, W., Koley, H., & Gachhui, R. (2016). Antibacterial activity of polyphenolic fraction of kombucha against enteric bacterial pathogens. *Current Microbiology*, 73, 885–896.
6. Butt, M. S., Ahmed, A., Sultan, M. T., Imran, A., Yasin, M., & Imran, M. (2011). Evaluating the effect of decaffeination on nutritional and antioxidant status of different coffee brands. *Internet Journal of Food Safety*, 13(1), 198–207.
7. Chakravorty, S., Bhattacharya, S., Chatzinotas, A., Chakraborty, W., Bhattacharya, D., & Gachhui, R. (2016). Kombucha tea fermentation: Microbial and biochemical dynamics. *International Journal of Food Microbiology*, 220, 63–72.
8. Chen, C., & Liu, B. Y. (2000). Changes in major components of tea fungus metabolites during prolonged fermentation. *Journal of Applied Microbiology*, 89(5), 834–839.
9. Cheung, L. M., Cheung, P. C. K., & Ooi, V. E. C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, 81(2), 249–255.
10. Chu, S.-C., & Chen, C. (2006). Effects of origins and fermentation time on the antioxidant activities of kombucha. *Food Chemistry*, 98(3), 502–507.
11. Coton, M., Pawtowski, A., Taminiau, B., Burgaud, G., Deniel, F., Coulloume-Labarthe, L., Fall, A., Daube, G., & Coton, E. (2017). Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods. *FEMS Microbiology Ecology*, 93(5).
12. Cushnie, T. P. T., & Lamb, A. J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents*, 38(2), 99–107.
13. Czaja, W., Krystynowicz, A., Bielecki, S., & Brown Jr, R. M. (2006). Microbial cellulose—the natural power to heal wounds. *Biomaterials*, 27(2), 145–151.
14. Czekanska, E. M. (2011). Assessment of cell proliferation with resazurin-based fluorescent dye. *Mammalian Cell Viability: Methods and Protocols*, 27–32.
15. D'Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., & Masella, R. (2007). Polyphenols, dietary sources and bioavailability. *Annali-Istituto Superiore Di Sanita*, 43(4), 348.

16. Dufresne, C., & Farnworth, E. (2000). Tea, Kombucha, and health: a review. *Food Research International*, 33(6), 409–421.
17. Farah, A. (2012). Coffee: emerging health effects and disease prevention. *Coffee Constituents*, 21–58.
18. Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, 408(6809), 239–247.
19. Floegel, A., Kim, D.-O., Chung, S.-J., Koo, S. I., & Chun, O. K. (2011). Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *Journal of Food Composition and Analysis*, 24(7), 1043–1048.
20. Fukagawa, S., Haramizu, S., Sasaoka, S., Yasuda, Y., Tsujimura, H., & Murase, T. (2017). Coffee polyphenols extracted from green coffee beans improve skin properties and microcirculatory function. *Bioscience, Biotechnology, and Biochemistry*, 81(9), 1814–1822.
21. Gómez-Ruiz, J. Á., Leake, D. S., & Ames, J. M. (2007). In vitro antioxidant activity of coffee compounds and their metabolites. *Journal of Agricultural and Food Chemistry*, 55(17), 6962–6969.
22. Greenwalt, C. J., Steinkraus, K. H., & Ledford, R. A. (2000). Kombucha, the fermented tea: microbiology, composition, and claimed health effects. *Journal of Food Protection*, 63(7), 976–981.
23. Hara, Y. (1995). Processing of tea. *Food Rev. Int.*, 11, 426–430.
24. Ivanišová, E., Meňhartová, K., Terentjeva, M., Godočíková, L., Árvay, J., & Kačániová, M. (2019). Kombucha tea beverage: microbiological characteristic, antioxidant activity, and phytochemical composition. *Acta Alimentaria*, 48(3), 324–331.
25. Jayabalan, R., Malbaša, R. V., Lončar, E. S., Vitas, J. S., & Sathishkumar, M. (2014). A review on kombucha tea—microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. *Comprehensive Reviews in Food Science and Food Safety*, 13(4), 538–550.
26. Jayabalan, R., Marimuthu, S., & Swaminathan, K. (2007). Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. *Food Chemistry*, 102(1), 392–398.
27. Kaneko, S., Chen, J., Wu, J., Suzuki, Y., Ma, L., & Kumazawa, K. (2017). Potent odorants of characteristic floral/sweet odor in Chinese chrysanthemum flower tea infusion. *Journal of Agricultural and Food Chemistry*, 65(46), 10058–10063.
28. Keflie, T. S., Nölle, N., Lambert, C., Nohr, D., & Biesalski, H. K. (2019). Impact of the natural resource of UVB on the content of vitamin D2 in oyster mushroom (*Pleurotus ostreatus*) under subtropical settings. *Saudi Journal of Biological Sciences*, 26(7), 1724–1730.
29. Kumar, V., & Joshi, V. K. (2016). Kombucha: Technology, microbiology, production, composition and therapeutic value. *International Journal of Food and Fermentation Technology*, 6(1), 13–24.
30. Lo, K. M., & Cheung, P. C. K. (2005). Antioxidant activity of extracts from the fruiting bodies of *Agrocybe aegerita* var. *alba*. *Food Chemistry*, 89(4), 533–539.
31. Lopez, C. L. F., Beaufort, S., Brandam, C., & Taillandier, P. (2014). Interactions between *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* in tequila must type medium fermentation. *World Journal of Microbiology and Biotechnology*, 30, 2223–2229.

32. Malbaša, R. V, Lončar, E. S., Vitas, J. S., & Čanadanović-Brunet, J. M. (2011). Influence of starter cultures on the antioxidant activity of kombucha beverage. *Food Chemistry*, 127(4), 1727–1731.
33. Marsh, A. J., O'Sullivan, O., Hill, C., Ross, R. P., & Cotter, P. D. (2014). Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. *Food Microbiology*, 38, 171–178.
34. Mo, H., Zhu, Y., & Chen, Z. (2008). Microbial fermented tea—a potential source of natural food preservatives. *Trends in Food Science & Technology*, 19(3), 124–130.
35. Mukkundur Vasudevaiah, A., Chaturvedi, A., Kulathooran, R., & Dasappa, I. (2017a). Effect of green coffee extract on rheological, physico-sensory and antioxidant properties of bread. *Journal of Food Science and Technology*, 54, 1827–1836.
36. Mukkundur Vasudevaiah, A., Chaturvedi, A., Kulathooran, R., & Dasappa, I. (2017b). Effect of green coffee extract on rheological, physico-sensory and antioxidant properties of bread. *Journal of Food Science and Technology*, 54, 1827–1836.
37. Nakayama, G. R. (1997). Assessment of the Alamar Blue assay for cellular growth and viability in vitro. *J Immunol Methods*, 204, 205–208.
38. Nazemi, L., Hashemi, S. J., Ghazvini, R. D., Saeedi, M., Khodavaisy, S., Barac, A., Modiri, M., Dana, M. A., & Rezaie, S. (2019). Investigation of cgrA and cyp51A gene alternations in *Aspergillus fumigatus* strains exposed to kombucha fermented tea. *Current Medical Mycology*, 5(3), 36.
39. Nguyen, N. K., Nguyen, P. B., Nguyen, H. T., & Le, P. H. (2015). Screening the optimal ratio of symbiosis between isolated yeast and acetic acid bacteria strain from traditional kombucha for high-level production of glucuronic acid. *LWT-Food Science and Technology*, 64(2), 1149–1155.
40. Nwachukwu, E., & Uzoeto, H. O. (2010). Antimicrobial activity of some local mushrooms on pathogenic isolates. *Journal of Medicinal Plants Research*, 4(23), 2460–2465.
41. O'brien, J., Wilson, I., Orton, T., & Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry*, 267(17), 5421–5426.
42. Padmini, E., Valarmathi, A., & Rani, M. U. (2010). Comparative analysis of chemical composition and antibacterial activities of *Mentha spicata* and *Camellia sinensis*. *Asian J. Exp. Biol. Sci*, 1(4), 772–781.
43. Perdani, C. G., & Pranowo, D. (2019). Total phenols content of green coffee (*Coffea arabica* and *Coffea canephora*) in East Java. *IOP Conference Series: Earth and Environmental Science*, 230(1), 012093.
44. Podolich, O., Kukharensko, O., Haidak, A., Zaets, I., Zaika, L., Storozhuk, O., Palchikovska, L., Orlovska, I., Reva, O., & Borisova, T. (2019). Multimicrobial kombucha culture tolerates Mars-like conditions simulated on low Earth orbit. *Astrobiology*, 19(2), 183–196.
45. Repetto, G., & Sanz, P. (1993). Neutral red uptake, cellular growth and lysosomal function: in vitro effects of 24 metals. *Alternatives to Laboratory Animals*, 21(4), 501–507.
46. Ribeiro, L. S., Miguel, M. G. da C. P., Evangelista, S. R., Martins, P. M. M., van Mullem, J., Belizario, M. H., & Schwan, R. F. (2017). Behavior of yeast inoculated

- during semi-dry coffee fermentation and the effect on chemical and sensorial properties of the final beverage. *Food Research International*, 92, 26–32.
47. Rice-Evans, C., Miller, N., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, 2(4), 152–159.
 48. Rojas, A., Hernandez, L., Pereda-Miranda, R., & Mata, R. (1992). Screening for antimicrobial activity of crude drug extracts and pure natural products from Mexican medicinal plants. *Journal of Ethnopharmacology*, 35(3), 275–283.
 49. Roshan, H., Nikpayam, O., Sedaghat, M., & Sohrab, G. (2018). Effects of green coffee extract supplementation on anthropometric indices, glycaemic control, blood pressure, lipid profile, insulin resistance and appetite in patients with the metabolic syndrome: a randomised clinical trial. *British Journal of Nutrition*, 119(3), 250–258.
 50. Santos Jr, R., Batista, R. A., & Rodrigues, S. A. (2009). LX Filho,“. *Antimicrobial Activity of Broth Fermented with Kombucha Colonies*,” *J Microbial Biochem Technol*, 1(1), 72–78.
 51. Sen, S., Chakraborty, R., Sridhar, C., Reddy, Y. S. R., & De, B. (2010). Free radicals, antioxidants, diseases and phytomedicines: current status and future prospect. *International Journal of Pharmaceutical Sciences Review and Research*, 3(1), 91–100.
 52. Sezer, Ç., & Güven, A. (2009). Investigation of bacteriocin production capability of lactic acid bacteria isolated from foods. *Kafkas Univ Vet Fak Derg*, 15(1), 45–50.
 53. Shebis, Y., Iluz, D., Kinel-Tahan, Y., Dubinsky, Z., & Yehoshua, Y. (2013). *Natural antioxidants: function and sources*.
 54. St-Pierre, D. L. (2019). *Microbial diversity of the symbiotic colony of bacteria and yeast (SCOBY) and its impact on the organoleptic properties of kombucha*. The University of Maine.
 55. Su, W.-Y., Gao, S.-Y., Zhan, S.-J., Wu, Q., Chen, G.-M., Han, J.-Z., Lv, X.-C., Rao, P.-F., & Ni, L. (2022). Evaluation of volatile profile and in vitro antioxidant activity of fermented green tea infusion with pleurotus sajor-caju (Oyster Mushroom). *Frontiers in Nutrition*, 9.
 56. Talawat, S., Ahantharik, P., Laohawiwattanakul, S., Premasuk, A., & Ratanapo, S. (2006). Efficacy of fermented teas in antibacterial activity. *Agriculture and Natural Resources*, 40(4), 925–933.
 57. Tang, S., Tu, C., Hu, W., & Dong, M. (2019). Antioxidant activity of fermented soy whey with kombucha consortium. *Shipin Kexue/Food Science*, 40(17), 1–6.
 58. Villarreal-Soto, S. A., Beaufort, S., Bouajila, J., Souchard, J., & Taillandier, P. (2018a). Understanding kombucha tea fermentation: a review. *Journal of Food Science*, 83(3), 580–588.
 59. Villarreal-Soto, S. A., Beaufort, S., Bouajila, J., Souchard, J., & Taillandier, P. (2018b). Understanding kombucha tea fermentation: a review. *Journal of Food Science*, 83(3), 580–588.
 60. Vitas, J. S., Malbaša, R. V, Grahovac, J. A., & Lončar, E. S. (2013). The antioxidant activity of kombucha fermented milk products with stinging nettle and winter savory. *Chemical Industry and Chemical Engineering Quarterly*, 19(1), 129–139.
 61. Vohra, B. M., Fazry, S., Sairi, F., & Babul-Airianah, O. (2019a). Effects of medium variation and fermentation time on the antioxidant and antimicrobial properties of

- Kombucha. *Malaysian Journal of Fundamental and Applied Sciences*, 15(2–1), 298–302.
62. Vohra, B. M., Fazry, S., Sairi, F., & Babul-Airianah, O. (2019b). Effects of medium variation and fermentation time on the antioxidant and antimicrobial properties of Kombucha. *Malaysian Journal of Fundamental and Applied Sciences*, 15(2–1), 298–302.
 63. Watawana, M. I., Jayawardena, N., Gunawardhana, C. B., & Waisundara, V. Y. (2016). Enhancement of the antioxidant and starch hydrolase inhibitory activities of king coconut water (*Cocos nucifera* var. *aurantiaca*) by fermentation with kombucha 'tea fungus.' *International Journal of Food Science & Technology*, 51(2), 490–498.
 64. Watawana, M. I., Jayawardena, N., & Waisundara, V. Y. (2015). Enhancement of the Functional Properties of Coffee Through Fermentation by "Tea Fungus"(Kombucha). *Journal of Food Processing and Preservation*, 39(6), 2596–2603.
 65. Wolfe, B. E., & Dutton, R. J. (2015). Fermented foods as experimentally tractable microbial ecosystems. *Cell*, 161(1), 49–55.
 66. Wong, K. H., Sabaratnam, V., Abdullah, N., Kuppusamy, U. R., & Naidu, M. (2009). Effects of cultivation techniques and processing on antimicrobial and antioxidant activities of *Hericium erinaceus* (Bull.: Fr.) Pers. extracts. *Food Technology and Biotechnology*, 47(1), 47–55.
 67. Wu, W.-J., & Ahn, B.-Y. (2014). Statistical optimization of ultraviolet irradiate conditions for vitamin D2 synthesis in oyster mushrooms (*Pleurotus ostreatus*) using response surface methodology. *PLoS One*, 9(4), e95359.
 68. Zhang, J., Van Mullem, J., Dias, D. R., & Schwan, R. F. (2021). The chemistry and sensory characteristics of new herbal tea-based kombuchas. *Journal of Food Science*, 86(3), 740–748.
 69. Zhao, C.-N., Tang, G.-Y., Cao, S.-Y., Xu, X.-Y., Gan, R.-Y., Liu, Q., Mao, Q.-Q., Shang, A., & Li, H.-B. (2019). Phenolic profiles and antioxidant activities of 30 tea infusions from green, black, oolong, white, yellow and dark teas. *Antioxidants*, 8(7), 215.
 70. Zofia, N.-Ł., Aleksandra, Z., Tomasz, B., Martyna, Z.-D., Magdalena, Z., & Zofia, H.-B. (2020). Effect of fermentation time on antioxidant and anti-ageing properties of green coffee Kombucha ferments. *Molecules*, 25(22), 5394.