Malaysian Journal of Chemistry, 2024, Vol. 26(5), 294-305
(Special Issue: *3rd Virtual International Symposium on Sciences 2023*)

Optimising the Nutritional Value of Kombucha Using Fruit Peels as Functional Ingredients

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Kombucha, a popular fermented beverage made from tea leaves, has not been extensively studied in terms of the incorporation of fruit peels that are often wasted. This research aims to produce and optimise a kombucha beverage by utilising fruit peels, specifically dried orange (*Citrus reticulata*) peels infusions formulated with dried pomegranate (*Punica granatum* L.) peels, as functional food ingredients. Eight kombucha formulations infused with orange peel (OP) and pomegranate peel (PP) were obtained from a simplex lattice mixture design (Design Expert 13.0.1 software). The optimisation parameters for formulating the kombucha were total phenolic content (TPC), antioxidant activity using DPPH radical scavenging activity assay, and antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* using well diffusion assay. An optimal formulation of kombucha incorporated with OP and PP was obtained and the results demonstrated that the optimised formulation consisted of 32.40% OP and 67.60% PP, achieving a desirability value of 0.505. This combination of OP and PP improved the TPC, antioxidant activity and antimicrobial activity of the optimised kombucha. This study presents an optimised formulation and highlights the potential of incorporating fruit peels to enhance the nutritional value of kombucha with new flavours. The newly formulated kombucha may offer new variations of kombucha flavour compared to commercialised products in future studies.

Keywords: Kombucha; orange peels; pomegranate peels; optimisation; Design Expert

Received: November 2023; Accepted: February 2024

One of the most important fruit crops in the world is citrus, which belongs to the genus *Citrus reticulata* in the family Rutaceae. It is mainly grown throughout the world's tropical and subtropical regions and many other places, producing over 102 million tons yearly [1]. Citrus fruits are primarily utilised in the juice processing industry. Therefore, a tremendous amount of trash orange by-products such as peels are generated every year [2]. In the orange industry, the waste with the highest volume and greatest value is orange peel, which makes up about 20% of an orange. The estimated amount of orange peel waste generated in 2018 is 15.10 metric tons [3]. Citrus fruit peels, which contain several secondary components with high antioxidant activity compared to other fruit components, are commonly wasted because citrus fruits are widely consumed worldwide as fresh produce, i.e., citrus juice [4]. Citrus peels can be used effectively as food supplements or medicines because they contain nutrients and phytochemicals [2]. Orange peel waste is a rich source of essential ingredients that can be used to make bioproducts with a high level of added value [5]. Based on data cited and collected by previous study [3], not all orange peel waste is utilised, leaving non-hazardous waste possibilities for revalorization.

An ancient fruit called the pomegranate (*Punica granatum* L.) is primarily grown in west Asia, though it is also grown in the Mediterranean region and other places worldwide. The fruit's edible part is found in the arils. Pomegranate exocarp, or the peel, makes up about 50% of the whole fruit, while the edible portion comprises 10% seeds and 40% arils [6]. Its positive physiological activities, particularly its antioxidant, antibacterial, and anti- inflammatory capabilities, have recently been discovered in vitro and in vivo research [7]. The use of pomegranate and its extracts (primarily as antioxidants and antimicrobials) in many food items has been thoroughly explored with positive outcomes, in line with this trend and the food industry's demand for antioxidants and antimicrobials from natural sources [8]. With the increasing demand for pomegranate and its extracts, especially for their antioxidant and antimicrobial properties [8], more pomegranates are processed. This processing leads to a larger quantity of by-products being produced, particularly the peel, which makes up about half of the entire fruit. These

†Paper presented at the 3rd Virtual International Symposium on Sciences (3rd VISoS 2023)

peels can then be used for various purposes, such as extracting bioactive compounds or creating dietary supplements. Therefore, production of its primary by-products such as peels increased.

The fruit processing industry plays a vital role in meeting global demand for fruit products, yet it generates significant quantities of by-products. These by-products are often considered waste and disposed of, leading to environmental pollution, resource depletion, and missed opportunities for value creation. However, the growing emphasis on sustainable practices and the concept of circular economy highlights the need for efficient utilisation of fruit by-products [9]. Fruit by-products pose several challenges that necessitate effective management strategies. Firstly, their improper disposal can contribute to environmental pollution, including soil and water contamination [10]. Additionally, their accumulation in landfills leads to increased greenhouse gas emissions, contributing to climate change [9]. Furthermore, the disposal of fruit by-products often incurs significant costs for processing industries, affecting their profitability and sustainability [3]. Addressing these challenges requires exploring alternative utilisation methods for fruit by-products. Various utilisation opportunities exist for fruit by-products, offering potential benefits to both the environment and the economy. The utilisation strategies encompass different sectors and value chains [11]. For instance, fruit peels can be transformed into animal feed, composted for soil enrichment, or used for essential oil extraction [9].

Nowadays, kombucha is consumed by a large population due to its health benefits. The biotransformation of sugared tea creates this healthy beverage via yeasts, fungi, and bacteria that produce acetic acid. The word "kombucha" has its roots in tea (cha) and Japanese seaweed (kombu). The kombucha's microbial composition is influenced by the types of yeasts and bacteria used, the environmental and climatic conditions in which they were grown, and its starting microbiome [12]. It has long been recognised that kombucha tea provides revitalising and cleansing effects. Additionally, kombucha tea was first ingested in East Asia for its curative properties, including for treating digestive issues. Additionally, kombucha's popularity skyrocketed as it is promoted as a healthy fermented food with benefits comparable to yoghurt consumption [13]. In general, the first step in preparing kombucha beverages entails the brewing method. Most kombucha formulas use tea as the primary infusion, including black tea, green tea, oolong tea, and others. Commonly, tea leaves are infused into freshly boiled water for about 10 minutes with the addition of sweetener. After removing the tea leaves, the tea is allowed to cool to room temperature and a starter culture, i.e., a symbiotic culture of bacteria and yeast (SCOBY) is added. The SCOBY is composed of cellulose layer and sour broth and is kept in a refrigerator (4ºC) (SCOBY floating on the liquid surface) [14]. Approximately, 1 L distilled water (90°C) is combined with 100 g of sugar (100g/L, 10.0%) and 10 g of tea leaf (10 g/L,10%) for the brewing method [14, 15]. To the author's best knowledge, no scientific study has been published on dried orange peel (OP) infusions formulated with dried pomegranate peel (PP) as the substrate for kombucha fermentation. In the present research, the mixture of dried OP and PP were used to replace the tea leaf as the medium of infusion. Fruit peels are frequently discarded as food waste. By using them in kombucha production, it can reduce waste and contribute to a more sustainable approach. Repurposing fruit peels aligns with the principles of environmental responsibility and can be an effective way for utilising a by-product that would otherwise go to waste, considering that previous studies had highlighted the benefits and bioactive components contained in fruit peels. In addition, several types of beverages, drinks, and related products primarily based on fruits had been developed using the mixture design technique [16]. Therefore, the dried orange and pomegranate peels kombucha beverages in this study were optimised based on their total phenolic content (TPC), antioxidant activity and antimicrobial activity using mixture design (Design Expert software).

EXPERIMENTAL

Chemicals and Materials

Sodium hydroxide, sodium bicarbonate (food grade), 1% phenolphthalein, sodium carbonate, gallic acid, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), nutrient agar (NA) powder, nutrient broth (NB) powder, 95% ethanol, and quercetin were obtained from UiTM Cawangan Negeri Sembilan, Kampus Kuala Pilah laboratory.

Preparation of Orange Peel and Pomegranate Peel Powders

Both orange and pomegranate fruits were obtained from a supermarket in Kuala Pilah, Negeri Sembilan, Malaysia. The selection of the fruits was based on their colour, i.e., orange for orange fruit and reddish brown for pomegranate fruit. The preparation of OP powder was conducted according to previous study [17] with some modifications. Meanwhile, PP powder was prepared with some alterations, in accordance with previous studies [18, 19]. The orange and pomegranate fruits were washed with distilled water to get rid of the dust particles. The OP and PP were separated from the pulp and the peels were cut into small pieces. Both peels underwent the debittering method and sterile treatment [20]. The peels were boiled in a 2% sodium bicarbonate ($NaHCO_3$) solution for 20 minutes. The peels were dried in a drying cabinet at 60°C for 4 days (OP) and 2 days (PP). After drying, the colour of OP turned to a brownish tone (brownish orange colour) while PP turned into a dark brownish red colour. The dried OP and PP were

crushed to powder using a grinder (30000 rpm), and the OP and PP powder were sieved through 355 µm stainless steel mesh. The OP and PP powder were then stored in a glass bottle for further analysis.

Optimisation of Kombucha Beverage Formulations using Simplex-Lattice Mixture Design

The percentage of dried OP and PP incorporated in the kombucha beverage was optimised using Design Expert software (Stat-Ease, Inc., Version 13.0, Minneapolis, USA).

Experimental Design

A two-component constrained simplex-lattice mixture design was performed using Design Expert software. Table 1 shows the experimental design for a percentage of OP and PP powders obtained from the simplex-lattice mixture design.

The experimental design consisting of eight points was developed to determine the combined effect of OP powder (X_1) and PP powder (X_2) as the two factors of independent variables. The experimental domain consisted of different proportions of independent variables between zero and one ($0 \leq X \leq 1$). To allow for error estimation, all the mixtures were prepared in two independent replications. As dependent variables, TPC, antioxidant activity (DPPH radical scavenging activity) and antimicrobial activity were chosen.

Preparation of Kombucha Beverages

In order to prevent contamination from airborne moulds or pathogenic organisms, sterile containers and utensils must be used during the preparation of kombucha beverages. The mixture of OP and PP were prepared according to the percentages shown in Table 1. In this study, all 8 formulations of kombucha beverages were prepared using a steeping method along with the control kombucha formulation (without the addition of fruit peels). The OP and PP powders (10 g) were placed into a small bag (tea bag size) and steeped in a sterile conical flask with 1 L of boiling water (100°C) for 10 minutes. The small bag was removed, and the solution was allowed to cool down before adding the sugar (10%), sour broth (10%) and SCOBY (50 g). The sour broth was prepared and fermented 14 days earlier using 10% of sugar in 1 L of water and SCOBY (50 g). The control sample was prepared using the same ingredients as the other formulations, with the exception of the fruit peel powder. The solution was transferred into clean glass bottles after being filtered with nylon filters and allowed to ferment for 14 days. The selection of a 14-day fermentation period for kombucha is rooted in previous research which suggests that this timeframe optimally cultivates its desired attributes. This process entails a dynamic equilibrium among diverse bacterial and yeast strains inherent in the SCOBY. A 14-day fermentation regimen aids in the establishment and sustenance of this microbial equilibrium, which is crucial for the development of flavour profile and potential health advantages associated with kombucha [12].

The SCOBY's continual metabolism of sugars and nutrients during fermentation underscores the significance of this period. It ensures sufficient time for the SCOBY to fully utilise available nutrients, culminating in a beverage of superior quality. The previous study [21] indicates a correlation between fermentation duration and the increase in phenolic content and antioxidant activity. This prolonged fermentation period further enriches kombucha's flavour profile, accentuating not only its tangy acidity but also the emergence of diverse esters, phenolic compounds, and other aromatic elements. All these collectively contribute to the distinctive taste and aroma profile of kombucha [12].

Table 1. Experimental Design for Percentage of Orange Peel and Pomegranate Peel Powders obtained from Simplex-Lattice Mixture Design.

Sample No	Factors (%)	
	X_1	X_2
1	0.25	0.75
2	1.00	0
3	0	1.00
4	0	1.00
5	0.5	0.5
6	0.75	0.25
7	0.5	0.5
8	1.00	0

Note: X_1: Dried Orange Peel, X_2: Dried Pomegranate Peel

Determination of Optimised Kombucha Beverage Formulations

Three parameters including TPC, antioxidant activity (DPPH radical scavenging activity) and antimicrobial activity of the kombucha beverages were determined at day 0, day 7 and day 14 of fermentation for optimisation of kombucha beverage formulations.

Determination of Total Phenolic Content (TPC)

The TPC of the kombucha beverages was analysed using the Folin-Ciocalteu reagent and gallic acid as the standard according to the method by previous study [22]. Approximately 10 mg of gallic acid was dissolved in 10 mL of methanol to produce a standard stock solution (1 mg/mL). The standard stock solution was diluted to prepare various concentrations of gallic acid solutions (0, 20, 40, 60, 80 and 100 µg/mL). A test tube containing 0.1 mL of sample and standard solution was added with 0.5 mL of 50% Folin-Ciocalteau reagent and 7.9 mL of distilled water. Then the mixture was vortexed for 15 seconds and left at room temperature for 5 minutes. Next, 1.5 mL of 0.57 M Na2CO3 (in which 3.021 g of Na2CO3 was used to produce 50 mL of 0.57 M sodium carbonate solutions) was added, and the test tubes were left to stand for 2 hours. The absorbance reading of all the sample and standard mixture were measured using UV-Vis Spectrophotometer at 750 nm, and distilled water was used as the blank. TPC was expressed as mg GAE (Gallic Acid Equivalent)/mL.

Determination of Antioxidant Activity

DPPH radical scavenging activity assay was used according to the method by previous study [23] to determine the antioxidant activity of kombucha beverages. Approximately 7.89 mg of DPPH was dissolved in 100 mL of 95% ethanol, stirred using a magnetic stirrer and placed in an Amber Schott bottle. Then, 0.6 mL of the kombucha sample was mixed with 4.5 mL of a 0.1 mM DPPH solution. After 30 minutes of dark incubation, the absorbance of the mixture was measured using UV-Vis Spectrophotometer at 517 nm and the sample blank was used as the control. Antioxidant activity was assessed by measuring changes in absorbance, and the percentage of DPPH radical scavenging activity (%) was calculated by using the formula as below:

$$\text{Scavenging Activity (\%)} = [A^o - A^s / A^o] \times 100 \quad \text{(Equation 1)}$$

where:

A^o—absorbance of DPPH solution at 517 nm without tested sample (Blank)

A^s—absorbance of DPPH solution at 517 nm with tested sample

Determination of Antimicrobial Activity

Antimicrobial activity of the kombucha beverages was determined using well diffusion assay against two bacteria: *Staphylococcus aureus* and *Escherichia coli*.

Preparation of nutrient agar plate: Approximately 28 g of NA powder was mixed with 1 L of distilled water and the solution was heated to completely dissolve the medium. The NA was sterilised by autoclaving them for 15 minutes at 121°C. The sterile NA was poured to the sterile petri dish (4 mm deep) and allowed to cool. The media must be moist but should not have any observable water droplets.

Preparation of nutrient broth: Approximately 13 g of NB powder was mixed with 1 L of distilled water and the solution was heated to completely dissolve the medium. The NB was sterilised by autoclaving them for 15 minutes at 121°C.

Preparation of test microorganisms and microbial culture: Pure culture of *S. aureus* and *E. coli* isolates was sub-cultured onto NA using the streak plate method. An inoculating loop was sterilised by putting the loop into the flame until it is red hot and allowed to cool. The loop was inserted into the bacteria culture and removed some inoculum. Immediately, the inoculating loop was streaked very gently over the agar plate. The streaked plates were incubated at 37°C for 24 hours.

Well diffusion assay: The antibacterial activity using the well diffusion assay was conducted as cited in previous studies [23, 24] against two pathogenic bacteria namely *S. aureus* and *E. coli*. Active culture was prepared by inoculating pure colony or freshly cultured bacteria (24 hours) into NB and then incubated for 24 hours at 37 ± 2°C in an incubator shaker (200 rpm). The optical density (OD) of each culture was adjusted into 1 – 2 x 10^8 CFU/mL. The bacterial suspensions (100 µL) were transferred to the new agar plates and the entire NA surface was streaked using cotton swabs. The plates were rotated by 60° and the rubbing procedure was repeated. To allow for the absorption of extra moisture, the medium's surface was allowed to dry for 3 to 5 minutes, but no more than 15 minutes. A hole of 6 mm of agar well was made by using a cork borer. Then 100 µL of all 8 formulations including positive control and negative control was pipetted into the 6 mm agar well. Streptomycin was used as a positive control whilst kombucha without addition of peels was used as a negative control. All the plates were incubated at 37 ± 2°C for 24 hours. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zone in mm around the hole in each plate using a Vernier calliper.

Statistical Analysis

The Design Expert software (Stat-Ease, Inc., Version 13.0, Minneapolis, USA) was used for the experimental design and all the statistical evaluations of the results were evaluated using the Statistical Package Social Sciences (SPSS) software.

RESULTS AND DISCUSSION

Total Phenolic Content (TPC) of Kombucha Beverage Formulations

Kombucha, a fermented beverage, is known to contain a variety of bioactive compounds, including phenolic compounds. The TPC of kombucha refers to the collective concentration of phenolic compounds present in the beverage. Phenolic compounds are secondary metabolites that possess antioxidant properties and have been associated with potential health benefits [25]. They are widely distributed in plant-based foods and beverages, including tea, which serves as the base for kombucha production [26]. Table 2 shows the TPC of 8 kombucha beverage formulations.

All the formulations had significant differences ($p < 0.05$) from day 0 to day 7 of fermentation. All the formulations exhibited elevated TPC levels at the beginning of fermentation, specifically on day 0. However, as the fermentation progressed to day 7, a decline in TPC was observed across all formulations. Subsequently, by the end of fermentation on day 14, all the formulations, except for F2 (10.51 ± 0.05 mg GAE/mL) and F8 (11.69 ± 0.24 mg GAE/mL), displayed a significant decrease in TPC. In contrast, F2 and F8 exhibited a continuous decrease in TPC throughout the fermentation period. F1 showed the highest TPC reading (39.54 ± 0.46mg GAE/mL), while F8 showed the lowest reading (14.04 ± 0.22 mg GAE/mL). TPC values for all the formulations rapidly decreased on fermentation day 7, with F1 recording the highest (19.49 ± 0.73 mg GAE/mL) and F3 recording the lowest (8.67 ± 0.02 mg GAE/mL). When fermentation ended, F1 had the highest TPC among all the formulations (39.40 ± 0.38 mg GAE/mL), while F2 had the lowest TPC (10.51 ± 0.05 mg GAE/mL). The control sample showed an increase in TPC from day 0 to day 7, ranging from 5.72 ± 0.23 mg GAE/mL to 9.61 ± 0.37 mg GAE/mL. However, it subsequently decreased to 7.11 ± 0.02 mg GAE/mL on the final day of fermentation, i.e., day 14. Moreover, the formulation compositions and choice of OP or PP influenced the initial TPC values, with the PP-based formulations exhibiting higher TPC compared to the OP-based formulations. In comparison with the untreated sample (control), all treated samples incorporated with OP or PP exhibited a significant difference in TPC values.

Table 2. Total phenolic content of kombucha beverage formulations.

SAMPLE		TPC (mg GAE/mL)		
	FORMULATION	**Day 0**	**Day 7**	**Day 14**
Treated samples	**1**	39.54 ± 0.46^{Aa}	19.49 ± 0.73^{Ab}	39.40 ± 0.38^{Aa}
	2	17.69 ± 0.28^{Fa}	12.38 ± 0.61^{Cb}	10.51 ± 0.05^{Fc}
	3	29.67 ± 0.04^{Cb}	8.67 ± 0.02^{Fc}	32.66 ± 0.15^{Ba}
	4	26.81 ± 0.04^{Da}	10.31 ± 0.01^{Dc}	21.54 ± 0.47^{Db}
	5	27.56 ± 0.41^{Da}	11.74 ± 0.07^{Cb}	26.55 ± 0.33^{Ca}
	6	24.52 ± 0.10^{Ea}	11.54 ± 0.16^{Cc}	21.92 ± 0.10^{Db}
	7	30.25 ± 0.01^{Ba}	9.33 ± 0.09^{Ec}	20.59 ± 0.35^{Db}
	8	14.04 ± 0.22^{Ga}	12.47 ± 0.25^{Bb}	11.69 ± 0.24^{Ec}
Control sample		5.72 ± 0.23^{Hc}	9.61 ± 0.37^{Ea}	7.11 ± 0.02^{Gb}

Note: The different A-H capital letters indicate significant difference ($p < 0.05$) among formulation and control sample. The different a-c small letters indicate significant difference ($p < 0.05$) among fermentation days.

The variations observed in the TPC of the kombucha beverage formulations during the fermentation process can be attributed to multiple factors involved in fermentation. During the initial stage of fermentation (day 0), the steeping of OP and PP and the addition of the SCOBY may result in the release of phenolic compounds from the peels, leading to an increase in TPC. Subsequently, as fermentation proceeds, the microorganisms present in kombucha, such as yeast and bacteria, metabolise various compounds including phenolic compounds [12]. Through enzymatic processes, these microorganisms can break down or transform phenolic compounds, resulting in a decrease in TPC, as observed on day 7. As fermentation continues, the microorganisms further metabolise the substrates, giving rise to fermentation reactions that may involve the synthesis or reformation of phenolic compounds [27]. This can lead to an increase in TPC again, as observed on day 14. It is important to note that OP and PP used as substrates for kombucha production contain a complex mixture of phenolic compounds. Overall, the dynamic changes in TPC during kombucha fermentation reflect a complex interplay between microbial metabolism, chemical reactions, and the specific composition of phenolic compounds in the OP and PP. Further analysis and characterisation of individual phenolic compounds throughout the fermentation process would provide a more comprehensive understanding of their fate and contribution to the overall TPC fluctuations. In addition to tea polyphenols, the principal antioxidants in fermented kombucha tea beverages also include several tea fungal metabolites, including vitamins and organic acids [12]. Important extracellular enzymes involved in the structural modification of the molecules during kombucha fermentation also have an impact on the antioxidant capacity. As a result of this, kombucha-fermented tea typically exhibits greater antioxidant potential than non-fermented tea [12].

Antioxidant Activity of Kombucha Beverage Formulations

In the study, the antioxidant activity of the kombucha beverage formulations were determined using DPPH radical scavenging activity assay. Table 3 shows the DPPH radical scavenging activity (%) of 8 kombucha beverage formulations. All formulations show a significant difference in the amount of DPPH radical scavenging activity between treated samples and untreated samples (control), suggesting that the duration of fermentation and the composition of substrates made from a variety of OP and PP have an impact on the anti-radical properties of kombucha. In general, its ability to combat free radicals declined over the duration of the fermentation process for each kombucha formulation examined. These findings are consistent with the results reported by previous study [14], who noted a similar pattern of rising antioxidant potential (DPPH) during the early stages of fermentation, followed by a decrease in antioxidant potential after seven days of fermentation.

Table 3. DPPH radical scavenging activity (%) of kombucha beverage formulations.

SAMPLE / STANDARD		DPPH radical scavenging activity (%)		
	FORMULATION	**Day 0**	**Day 7**	**Day 14**
Treated samples	**1**	75.34 ± 1.10^{Eb}	88.08 ± 0.92^{Ba}	65.96 ± 1.13^{Cc}
	2	76.76 ± 0.64^{Ea}	71.02 ± 0.30^{Db}	50.50 ± 0.74^{Dc}
	3	77.84 ± 0.29^{Eb}	88.79 ± 0.52^{Ba}	70.50 ± 0.22^{Bc}
	4	82.11 ± 0.33^{Bb}	88.71 ± 0.23^{Ba}	70.68 ± 0.35^{Bc}
	5	81.00 ± 0.23^{Cb}	89.45 ± 0.42^{Ba}	70.58 ± 0.34^{Bc}
	6	80.49 ± 0.49^{Cb}	83.43 ± 0.20^{Ca}	70.67 ± 0.03^{Bc}
	7	79.56 ± 0.33^{Db}	90.01 ± 0.18^{Ba}	70.77 ± 0.23^{Bc}
	8	77.57 ± 0.30^{Ea}	66.82 ± 0.71^{Eb}	50.77 ± 0.03^{Dc}
Control sample		29.58 ± 1.12^{Fa}	24.74 ± 0.37^{Fb}	23.31 ± 3.61^{Ec}
Quercetin standard		98.09 ± 0.05^{Aa}	94.86 ± 0.01^{Ab}	96.21 ± 0.004^{Ac}

Note: The different A-F capital letters indicate significant difference ($p < 0.05$) among formulation, control sample and standard. The different a-c small letters indicate significant difference ($p < 0.05$) among fermentation days.

Based on the result, the 7th day of fermentation had the best effects on these antioxidant properties. The steeping method causes DPPH inhibition to increase all the formulations other than F2 and F8. Additionally, it was observed that on the 7th day of fermentation, both the microbial composition and the development of a new multilayer SCOBY were at their most diverse. This observation suggests that the increased diversity of microorganisms plays a significant role in enhancing the antioxidant properties of kombucha tea. Moreover, the shift from yeast dominance to lactic acid bacteria on the 7th day of fermentation is also responsible for the heightened antioxidant activity [14]. The presence of lactic acid bacteria further influenced the antioxidant activity of the beverage. The increased content of polyphenolic compounds can be attributed to various reactions occurring during the fermentation of fruit peels in kombucha, such as the enzymatic oxidation of polyphenols leading to the formation of flavonoids and other bioactive compounds including antioxidants as a result of microbial hydrolysis [12]. This may be the cause of some formulations that increased the percentage of radical scavenging activity on day 7.

The antioxidant activity of a substance is negatively affected by long heat exposure [28]. This is attributed to the partial degradation of bioactive compounds, particularly heat-sensitive antioxidants such as certain vitamins (e.g., vitamin C) and enzymes. During fermentation, the microorganisms presented in the kombucha consume nutrients and compounds available in the OP and PP, including antioxidants. As the fermentation progresses, the microorganisms utilise these antioxidants, leading to a decrease in the overall availability of antioxidant compounds, which can result in reduced DPPH radical scavenging activity [29]. The fermentation process of kombucha involves the conversion of various substrates into different metabolites. Some of these metabolites, such as organic acids, can exhibit their own antioxidant properties. However, other metabolites generated during fermentation may not contribute significantly to the antioxidant activity or may even interfere with the DPPH radical scavenging reaction, resulting in a decline in observed activity [29]. The phenolic composition of kombucha can undergo modifications during fermentation. Phenolic compounds, which are known for their antioxidant activity, may undergo degradation, transformation, or complexation with other compounds during the fermentation process. Byproducts produced during fermentation, such as alcohol, organic acids, and gases, can potentially interact with the DPPH radical scavenging reaction. These interactions may interfere with the assay and result in lower measured radical scavenging activity [29]. To highlight the presence of these specific compounds in orange peel and pomegranate peel, which are believed to contribute to the antioxidant activity in kombucha, it is noteworthy to mention that in orange peel, the primary phenolic compounds responsible for its antioxidant properties are flavanone glycosides. These flavanone glycosides, including naringin, hesperidin, narirutin, and neo hesperidin, are unique to citrus fruits and have demonstrated potent antioxidant effects. They are integral to the overall antioxidant capacity of orange peel extract [30]. Similarly, pomegranate peel contains significant amounts of phenolic acids such as hydroxycinnamic and hydroxybenzoic acids, along with a rich content of flavonoids. These flavonoids, such as anthocyanins, catechins, and other complex flavonoids, have been extensively studied for their antioxidant properties. It is believed that these compounds play a crucial role in the observed antioxidant activity of pomegranate peel extract, further enhancing the potential antioxidant benefits of kombucha enriched with pomegranate peel extract [31].

Antimicrobial Activity of Kombucha Beverage Formulations

In this study, the antimicrobial activity of kombucha beverage formulations were determined using well diffusion assay. The zones of inhibition were measured to determine the antimicrobial activity [23]. Table 4 and Table 5 show the inhibition zone diameter of the 8 kombucha beverage formulations against *S. aureus* and *E. coli*, respectively.

As shown in Table 4, no inhibition zone was observed on day 0 for *S. aureus*. As fermentation progressed to day 7, all formulations showed inhibition zones ranging from 16 mm to 22 mm. The F4 demonstrated the highest inhibition zone diameter (22 mm), while F1, F2, and F6 exhibited the same diameter of inhibition zone (21 mm). Similarly, F3, F5, and F7 showed the same inhibition zone diameter of 20 mm, while F8 displayed the lowest inhibition zone (16 mm). On day 14, all the formulations showed inhibition zones ranging from 22 mm to 25 mm. F2 exhibited the largest inhibition zone with a diameter of 25 mm, while both F1 and F5 displayed the lowest inhibition zone diameter of 22 mm. Table 5 presents the results of the well diffusion assay conducted to assess the inhibition zone against *E. coli* for all the formulations. No inhibition zone was observed on day 0. As fermentation progressed to day 7, all the formulations showed inhibition zones ranging from 23 mm to 28 mm. F5 demonstrated the highest inhibition zone diameter of 28 mm, while F1, F3, and F4 exhibited the same diameter of inhibition zone of 23 mm. Similarly, F7 and F8 showed the same inhibition zone diameter of 28 mm. On day 14, all the formulations showed decreasing inhibition zones ranging from 18 mm to 25 mm. F6 exhibited the largest inhibition zone with a diameter of 25 mm, while F7 and F8 displayed an inhibition zone diameter of 23 mm. On the other hand, F1 showed the lowest diameter of inhibition zone of 18 mm.

Table 4. The inhibition zone diameter of kombucha beverage formulations against *S. aureus.*

SAMPLE / STANDARD		INHIBITION ZONE DIAMETER (mm)		
	FORMULATION	**Day 0**	**Day 7**	**Day 14**
Treated samples	**1**	0.0 ± 0.0^{Bc}	21.0 ± 0.3^{Cb}	22.0 ± 0.5^{Ea}
	2	0.0 ± 0.0^{Bc}	21.0 ± 0.3^{Cb}	25.0 ± 0.4^{Ba}
	3	0.0 ± 0.0^{Bc}	20.0 ± 0.1^{Db}	23.0 ± 0.0^{Da}
	4	0.0 ± 0.0^{Bc}	22.0 ± 0.0^{Bb}	24.0 ± 0.1^{Ca}
	5	0.0 ± 0.0^{Bc}	20.0 ± 0.1^{Db}	22.0 ± 0.0^{Ea}
	6	0.0 ± 0.0^{Bc}	21.0 ± 0.2^{Cb}	23.0 ± 0.1^{Da}
	7	0.0 ± 0.0^{Bc}	20.0 ± 0.0^{Db}	24.0 ± 0.0^{Ca}
	8	0.0 ± 0.0^{Bc}	16.0 ± 0.1^{Eb}	24.0 ± 0.1^{Ca}
Control sample		0.0 ± 0.0^{Bb}	0.0 ± 0.0^{Fb}	22.0 ± 0.1^{Ea}
Streptomycin standard		45.0 ± 0.0^{Aa}	45.0 ± 0.0^{Aa}	45.0 ± 0.0^{Aa}

Note: The different A-F capital letters indicate significant difference ($p < 0.05$) among formulation, control sample and standard. The different a-c small letters indicate significant difference ($p < 0.05$) among fermentation days.

Table 5. The inhibition zone diameter of kombucha beverage formulations against *E. coli.*

SAMPLE / STANDARD		INHIBITION ZONE DIAMETER (mm)		
	FORMULATION	**Day 0**	**Day 7**	**Day 14**
Treated samples	**1**	0 ± 0.0^{Bc}	23 ± 0.4^{Fb}	18 ± 0.2^{Gb}
	2	0 ± 0.0^{Bc}	24 ± 0.1^{Eb}	19 ± 0.2^{Fb}
	3	0 ± 0.0^{Bc}	23 ± 0.1^{Fb}	19 ± 0.1^{Fb}
	4	0 ± 0.0^{Bc}	23 ± 0.3^{Fb}	20 ± 0.1^{Eb}
	5	0 ± 0.0^{Bc}	28 ± 0.2^{Bb}	21 ± 0.2^{Db}
	6	0 ± 0.0^{Bc}	26 ± 0.3^{Cb}	25 ± 0.1^{Bb}
	7	0 ± 0.0^{Bc}	25 ± 0.2^{Db}	23 ± 0.2^{Cb}
	8	0 ± 0.0^{Bc}	25 ± 0.2^{Db}	23 ± 0.3^{Cb}
Control sample		0 ± 0.0^{Bc}	0 ± 0.1^{Gb}	18 ± 0.1^{Gb}
Streptomycin standard		35 ± 0.0^{Ac}	35 ± 0.2^{Ab}	35 ± 0.1^{Ab}

Note: The different A-G capital letters indicate significant difference ($p < 0.05$) among formulation and control sample. The different a-c small letters indicate significant difference ($p < 0.05$) among fermentation days.

OP and PP kombucha inhibited *S. aureus* (Gram-positive bacteria) and *E. coli* (Gram-negative bacteria). This indicates that the natural bioactive compounds of the OP and PP possess antibacterial activity, which agrees with the previous studies that compared the control and showed that all the formulations that were added with OP and PP inhibited S. aureus and *E. coli* as analysed by the well diffusion assay. The bioactivity of kombucha was enhanced by the fermentation, whereby the inhibition was pronounced and enhanced by the presence of organic acids produced by acetobacter in the kombucha consortium [22]. The antimicrobial activity of kombucha is largely attributable to the presence of organic acids, particularly acetic acid, which can inhibit several Gram-positive and Gram-negative microorganisms [22]. In this study, the impact of OP and PP on the antimicrobial activity of kombucha was examined. It is well known that the peels of oranges and pomegranates both contain bioactive substances that may have antimicrobial activity.

The results showed that kombucha formulations incorporating OP exhibited significant antimicrobial activity against the tested microorganisms. The secondary metabolites found in abundance in *Citrus reticulata* contribute to the pharmacological effects attributed to this plant. It is thought that tannins, saponins, phenolic chemicals, essential oils, and flavonoids are responsible for plants' antimicrobial properties [32]. The presence of bioactive compounds in OP, such as flavonoids and essential oils, may contribute to its antimicrobial properties [33]. These compounds have been reported to possess inhibitory effects against a wide range of pathogenic bacteria. Similarly, kombucha formulations with PP also demonstrated antimicrobial activity, although to a higher extent compared to OP. PP contains phenolic compounds, such as ellagitannins and punicalagins, which have been associated with antimicrobial properties [31]. However, the concentration and composition of these bioactive compounds may vary depending on the source and processing of the PP.

It was noted that there is absence of inhibition for some formulations against *S. aureus* and *E. coli.* During the initial stages of fermentation, the kombucha culture, which includes the SCOBY, was introduced into the peel's substrate. At this stage, the microorganisms in the kombucha culture may still be adapting to their new environment and may not have reached optimal growth and metabolic activity. Consequently, the production of antimicrobial compounds may be limited, resulting in the absence of inhibition zones on day 0 [34]. As fermentation progresses, the microorganisms in the kombucha culture adapted and proliferated, leading to increased metabolic activity [12]. This increased metabolic activity resulted in the production of various antimicrobial compounds, such as organic acids, enzymes, and bacteriocins [35]. The bioactive compounds in OP and PP exhibited inhibitory effects on the growth of microorganisms, leading to the appearance of inhibition zones on fermentation day 7.

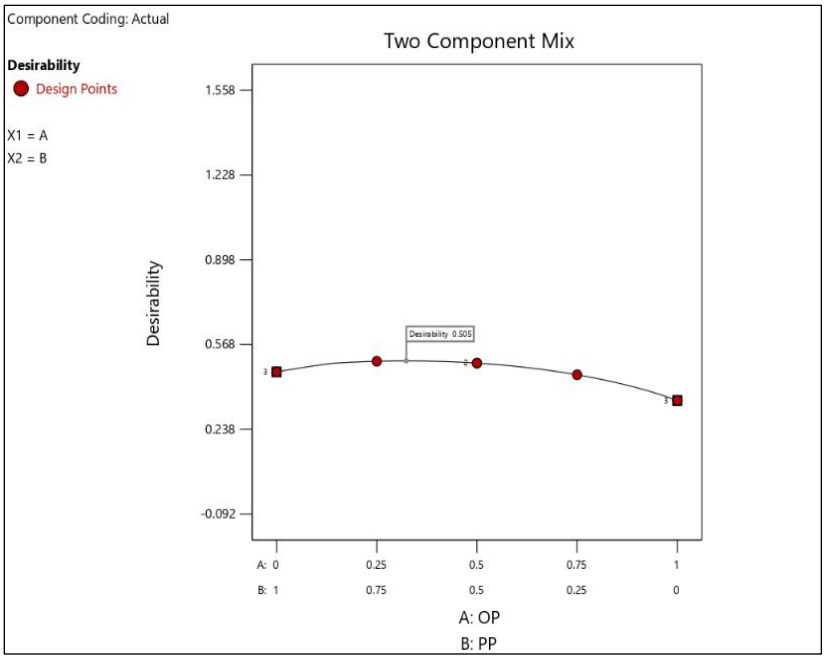
Several factors contribute to the decrease in inhibition zones at the end of fermentation. First, the availability of nutrients in the OP and PP substrates may become limited as the microorganisms consume and metabolise them. This nutrient limitation can impact the growth and metabolic activity of the microorganisms, including their ability to produce antimicrobial compounds. Second, the accumulation of metabolic byproducts during fermentation, such as organic acids, may alter the pH of the kombucha [36]. The shift towards a lower or higher pH range affects the antimicrobial activity of certain compounds, potentially reducing their inhibitory effects and leading to a decrease in inhibition zones [37]. Third, the population dynamics of the microorganisms in the kombucha culture may change over the course of fermentation. Some microorganisms may enter a stationary phase or decline in numbers, resulting in a decrease in their antimicrobial activity [37]. It is worth noting that the specific composition and activity of the microorganisms in the kombucha culture, as well as the initial OP and PP substrate and fermentation conditions, can influence the observed trends in the inhibition zones.

Optimised Kombucha Beverage Formulations

The optimisation of the kombucha beverage formulations was done based on their TPC, antioxidant activity and antimicrobial activity on day 14 using the simplex-lattice mixture design. This design approach allows for the systematic exploration and optimisation of multiple components or ingredients in a mixture, such as the proportions of orange and pomegranate peels used in the kombucha formulations. By using the simplex-lattice mixture design, the study aims to determine the optimal combination of OP and PP in the kombucha formulations that would result in desirable attributes or properties. This optimisation process involves selecting specific design points within the experimental space, conducting the brewing process for each formulation, and measuring the relevant response variables. Table 6 and Figure 1 show the optimised solution for kombucha beverage formulation.

Table 6. Optimised solution for kombucha beverage.

Number	OP	PP	TPC	DPPH	R3	R5	Desirability	
1	0.324	0.676	26.098	67.896	23.179	20.491	0.505	**Selected**



CONCLUSION

In conclusion, the optimised formulation consisted of 32.40% OP and 67.60% PP, with a desirability value of 0.505. These findings indicate the successful development of kombucha formulations incorporating dried orange and pomegranate peels which enhanced their total phenolic content, antioxidant, and anti-microbial activities.

ACKNOWLE.DGEMENTS

The authors would like to thank the Faculty of Applied Sciences, University Teknologi MARA Cawangan Negeri Sembilan, Kampus Kuala Pilah, Malaysia for the financial support and laboratory facilities.

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