PHYTOCHEMICAL SCREENING AND ANTIOXIDANT PROPERTIES OF BLACK AND GREEN KOMBUCHA TEA

Mahani Mahadi[a]*, Mohamad Syawal Mohd Zawawi[b], Zauyah Yusuf[c]

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Abstract: Kombucha tea is generally known as a fermented tea beverage yielded by fermenting sugar and black tea with a kombucha culture known as a mushroom. Due to its health benefit, this fermented tea had become one of popular drink worldwide. Kombucha tea has been reported to increase energy, promote detoxification, act as probiotic, possess anti-cancer, antioxidant, and anti-microbial properties. Therefore, this research aims to investigate the presence of phytochemical compounds and its antioxidant properties. In this study, green tea, black tea, sugar solution, black kombucha tea, green kombucha tea, and sugar kombucha solution as the tested group. All group was fermented in an incubator for 14 days and proceed for phytochemical screening and antioxidant analysis on day 0, 7 and 14. Identification alkaloids, flavonoids, phenol, saponin, tannin and glycosides were measured in each group. From the phytochemical analysis, black kombucha tea contain phenol, flavonoid and saponin meanwhile green kombucha tea contained phenol, flavonoid and tannin. Green and black tea contained phenol, flavonoid, saponin and tannin while sugar solution and sugar kombucha solution do not contain any phytochemical compound. Two antioxidant tests performed which are 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) scavenging assay and total phenolic compound (TPC). The result showed black and green kombucha tea, and sugar kombucha solution had the highest percentage of scavenging activity on day 7 and 14 compared to black and green tea and sugar solution. Similar to DPPH, highest TPC was observed in both black and green kombucha tea compared to other solutions. In conclusion, kombucha green and black tea contained several phytochemical compounds that may enhanced their antioxidant activity.

Keywords: Kombucha tea; antioxidant activity, metabolite profile

[a]. Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Cyberjaya, 63000 Cyberjaya, Selangor, Malaysia.
[b]. Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Cyberjaya, 63000 Cyberjaya, Selangor, Malaysia.
[c]. Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Cyberjaya, 63000 Cyberjaya, Selangor, Malaysia.

***Corresponding Author**

E-mail: mahani@cyberjaya.edu.my

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INTRODUCTION

Kombucha, is a traditional beverage with its slightly sour-sweet taste, has increased in popularity worldwide. To prepare kombucha tea, the sweetened black tea is fermented using a symbiotic culture of yeasts and bacteria. (Greenwalt et al., 2000). The beverage and the biofilm floating on the top of kombucha culture produces a mutual microbial community between bacteria and yeast (Chakravorty et al., 2016). There is an increased interest in kombucha as it has potential health effects. A kombucha tea that claimed to have a variety of health benefits is produced by fermenting sugared tea with a symbiotic culture of acetic acid bacteria and yeast. Studies claimed that consumption of kombucha tea has shown a significant effect in stimulating the immune system and liver function, a potential anticancer agent and able to reduced risk of cardiovascular disease (Jayabalan et al., 2014).

Practically, kombucha tea was fermented for 8 to 10 days, giving it a sour flavour that is similar to sparkling apple cider and gradually turns into a mild vinegar flavour over time (Marsh et al., 2014). The process of fermentation and oxidation started when the kombucha culture is placed in a freshly prepared infusion of tea and sugar. The process continued when the kombucha culture utilised the sucrose or the sugar and converted into monosaccharide form which is glucose and fructose. Ethanol and carbon dioxide were also being produce during this fermentation process with the presence of acetic acid bacteria in the medium (Kumar & Joshi, 2016). Kombucha tea was claimed to contain various components of organic acids such as malic, oxalic acids, tartaric malonic acid, acetic acid, L-lactic, an organic compound such as ethanol with other components includes sugars, amino acids, antibiotic active matters, and water-soluble vitamins. Moreover, study claimed that the kombucha tea was rich in mineral content such as Ni, K, Zn, Na, Ca, Cu and Fe (Kozyrovska et al., 2012). A similar study conducted by Goh et al., (2012) found that kombucha fermentation produces several metabolites that are obtained as a by-product includes vitamins Bs, monosaccharides such as fructose and various types of organic acids. The finding supported by the previous study conducted by Malbaša et al., (2011) claimed the kombucha tea has abundant of fibre, contains catechins, vitamin C, riboflavin (B_2), pyridoxin (B_6) and catalase, a dismutase enzyme that able to prevent oxidative damage. These properties

showed the potential of kombucha tea as antioxidative agent. Preventing oxidative damage and involves in cells signalling pathways are some of the functions of antioxidant in biological systems (Kumar, et al., 2008). An antioxidant is an element that holds or obstructs oxidation of any substrates (Halliwell, 2007), hence its prevention provides a multiple health benefit to the body. Vitamins (A, E, C), minerals (zinc and selenium), enzyme cofactors (Q10), nitrogen and phenolic compounds are some of known subgroup of antioxidant (Iluz et al., 2013).

The antioxidant properties of Kombucha tea influences by several parameters such as duration of fermentation, the type of tea use, and pH. Despite the growing popularity of this beverage, there is still a lack of information on the effects of tea varieties and their phytochemical compounds present. Therefore, this research aims to investigate the antioxidant properties and phytochemical screening from black, green and sugar kombucha teas at various time points of fermentation.

MATERIALS AND METHODS

Source of Kombucha: Kombucha starter culture was provided by Professor Dr Zauyah Yusuf, researcher of Faculty of pharmacy, University of Cyberjaya. Black and green tea (Lipton brand) and also table sugar from (Gula Perai) brand was purchased from the Mini-Mart (Speedmart), Cyberjaya, Selangor, Malaysia.

Methods

Tea Fungus Maintenance: The maintenance of the tea fungus was based on Goh et al., (2012). Briefly, 2000 mL of water was boiled before a sachet of black tea and 10 % of table sugar were added. This mixture was then stand at room temperature until cooled. Then, the mixture of sweetened tea was immediately transferred into a beaker after removing the tea bags. Finally, cellulosic pellicle fragments (3.0% w/v) and liquid broth (10% v/v) of the tea fungus sample were added into the cooled tea broth. The beakers were sealed by parafilm seal. The beaker then kept in the incubator for 14 days, at temperature (27 ± 3°C) to allow the fermentation process to take place.

Sample Grouping: Six groups of the sample were investigated which comprises of black tea, green tea, green kombucha tea, black kombucha tea, sugar kombucha solution and sugar solution. Sample from each group was taken at day 0, 7 and 14 days for analysis of antioxidant properties and phytochemical screening. Sampling was performed periodically, and the test was repeated for three times.

Sample Preparation: The preparation of kombucha tea is according to Jayabalan et al., (2008). 2000 mL of water was boiled and sterilized using an autoclave. Then 10% of table sugar and 6g of black and green tea were added to the beaker. According to Goh et al., (2012), acetic acid need to be added at the beginning of the fermentation process due to no accumulation of acids yet to form. Thus, this is to ensure no formation of moulds and undesirable microorganisms present in the tea broth. Finally, the freshly prepared tea was inoculated with 3% (W/V) of freshly kombucha mat that had been cultured and 10% (V/V) of previously fermented liquid broth. The beakers then were covered with parafilm seal. The fermentation was carried out under ambient temperature (27 ± 30C) for 14 days under aseptic conditions in the incubator. Black tea and green tea are prepared in beakers separately. All groups of the sample followed the same procedure. Sugar solution was prepared and used as a negative control. Black and green tea is used as a positive control. Fermented tea was collected at day 0, 7 and 14 and proceed with pH measurement. The tea was transfer into a sterile beaker and proceeded with the phytochemical screening and antioxidant activity test.

Antioxidant assay

DPPH Radical Scavenging Activity Assay: Free radical scavenging activity for each group against DPPH assay was evaluated spectrophotometrically based on Chan et al., (2007). 1 ml of sample was mixed with 2 ml of DPPH solution. The DPPH reagent was act as control while methanol solution act as a blank sample. The mixture was shaken and kept in dark for 30 minutes at room temperature. The reduction of DPPH free-radicals activity identifies by the measurement of absorbance at 517 nm. Ascorbic acid and Butylated Hydroxyanisole (BHA) were used as standards and the scavenging activity was calculated using the equation below:

$$RSA = \frac{Abs\ (control) - Abs\ (sample)}{Abs\ (control)} \times 100$$

where RSA refer to percentage of free radical scavenging activity, Abs (control) is the absorbance of DPPH only and Abs (sample) is the absorbance of DPPH radical with fermented tea or standard used.

Measurement of Total Phenolic Content (TPC): The total phenolic content measurement was conducted based on Chan et al., (2007). 1.5 ml of Folin-Ciocalteu reagent was diluted ten-fold was then added to 300 µL of the sample groups. The mixture was then incubated at room temperature for 3 minutes. Next, 1.2 ml sodium bicarbonate containing 75 g/L was added to the mixture and incubated for 30 minutes in the dark room. The absorbance was measured at 765 nm. Gallic acid was used as the standard and the phenolic compound content was expressed as gallic acid equivalent (GAE).

Series of dilution of Gallic acid was prepared for the TPC calibration curve. 1 ml aliquots of 0.2, 0.4, 0.6, 0.8 mg mL^{-1} ethanolic gallic acid solutions were mixed with 5 mL Folin-Ciocalteu reagent (diluted ten-fold) and 4 mL (75g/L) sodium bicarbonate (Miliauskas et al., 2004). After 30 minutes incubated in dark, the absorbance value was measured at 765 nm and the calibration curve was plotted. The concentration of phenolics (mg/mL) was then by comparing the standard prepared.

Phytochemical Screening

Test for alkaloid: Approximatively, 5 mL of the fermented tea was added with 1 mL of Mayer's reagent (Potassium Mercuric Iodide). The presence of yellow precipitate in the mixture indicates positive result for alkaloid.

Test for phenol- Ferric Chloride: About 5 mL of the fermented tea was carefully added with 3-4 drops of 5% ferric chloride solution. The presence of bluish black colour formation indicates the positive result for phenols.

Test for tannin-Gelatin: About 5 mL of the fermented tea was added with 3-4 drops of 1% ferric chloride solution. Presence of greenish colour formation indicates positive result for tannins.

Test for flavonoid-Alkaline Reagent: About 5 mL of the fermented tea was added with 3-4 drops of sodium hydroxide solution. Presence of intense yellow colour formation at the early mixture, which becomes colourless after the addition of diluted acid, indicates the presence of flavonoids.

Test for saponin-Froth: About 5 mL of the fermented tea was diluted with 15 ml of distilled water. The mixture was shaken slowly for 15 minutes. Presence of 1 cm layer of foam indicates the positive result for saponins.

Statistical analysis: All data were compiled in Microsoft Excel 2020 and undergo statistical analysis using SPSS version 23. The collected data are displayed as means ± standard error mean (SEM). The data was analysed using one-way ANOVA and it is considered as statistically significant if the p-values less than 0.05.

RESULTS

pH Measurement: pH measurement at day 0, 7 and 14 was shown on Table 3.1. From the table, black tea, green tea and sugar solution has a higher pH compared to green and black Kombucha tea and sugar Kombucha solution. The pH value was decreased during fermentation from day 0, day 7 to day 14 in all groups. No significant differences of pH value were observed between groups.

Table 3.1 Mean of pH among groups.

Groups	Mean± SEM		
	Day 0	Day 7	Day 14
Black Tea	5.68±0.130 [a]	3.26±0.150	2.93±0.024 [c]
Green Tea	6.02±0.076	2.87±0.150 [b]	2.05±0.020 [c]
Sugar Solution	6.15±0.100	3.83±0.370	3.65±0.058
Black Kombucha Tea	3.07±0.052 [a]	2.90±0.018 [b]	2.80±0.052 [c]
Green Kombucha Tea	3.07±0.042 [a]	2.77±0.022 [b]	2.64±0.026 [c]
Sugar Kombucha Solution	3.15±0.026 [a]	2.97±0.032 [b]	2.81±0.055 [c]

a: significant compared to green tea and sugar solution at day 0, $p<0.05$
b: significant compared to black tea and sugar solution at day 7, $p<0.05$
c: significant compared to sugar solution at day 14, $p<0.05$
Data expressed as mean±SEM.

Phytochemical screening: Table 3.2 showed the result of phytochemical screening of phenol, flavonoid, saponin, tannin and alkaloid in all samples. Phenol and flavonoid were detected in black tea, black kombucha tea, green tea and green kombucha tea. Both sugar and sugar kombucha solution do not show presence of any compounds. Saponin was only observed in black tea, black kombucha tea and green tea while tannin is detected high in green tea and little in black tea and green kombucha tea. No presence of alkaloid was detected in all groups**.**

Table 3.2 Summary of phytochemical screening result for sample groups.

Groups	Phenol	Flavanoid	Saponin	Tannin	Alkaloid
Black tea	++	++	+	+	-
Green tea	++	++	+	++	-
Sugar solution	-	-	-	-	-
Black kombucha tea	++	++	+	-	-
Green kombucha tea	++	++	-	+	-
Sugar kombucha solution	-	-	-	-	-

+ (intensity presence of phytochemical found on sample)
- (Show absent of phytochemical found on sample)

DPPH Radical Scavenging Activity Assay: Based on Table 3.3, DPPH radical scavenging activity at day 0 showed significant higher of activity in all groups compared sugar solution at $p< 0.05$. Similar pattern was observed in day 7. In day 14, all groups were significant with sugar and sugar kombucha solution at $p<0.05$. Black and green kombucha teas and sugar kombucha solution showed significant higher scavenging activity compared to black and green tea at $p<0.05$.

Table 3.3 The mean±SEM of percentage of scavenging activity between groups

Groups	Mean (%) ±SEM		
	Day 0	Day 7	Day 14
Black Tea	79.00±2.59[a]	85.88±0.43[a]	80.48±1.98 [a]
Green Tea	83.34±1.62 [a]	84.73±2.83[a]	77.34±1.53 [a]
Sugar Solution	61.89±2.80	66.35±1.59	48.60±3.87
Black Kombucha Tea	83.82±1.50 [a]	93.43±1.30 [a]	93.23±0.79 [a, c]

Green Kombucha Tea	83.88±1.61 [a]	92.53±0.88 [a]	94.96±1.10 [a, c]
Sugar Kombucha Solution	74.81±3.27 [a]	90.04±1.43 [a]	90.85±1.66 [a, c]

a: significant compared to sugar solution at $p<0.05$
b: significant compared to sugar solution at $p<0.05$
c: significant compared to green and black tea at $p<0.05$

Total Phenolic Content: The total phenolic content among groups was shown on Table 3.4. Black and green teas, black and green kombucha teas showed significant higher TPC compared to sugar solution and sugar kombucha solution at day 0 at $p<0.05$. Green kombucha tea has the highest TPC compared to all groups. At day 7 and day 14, similar pattern was observed. Black and green teas with or without kombucha showed significant higher TPC content compared to sugar and sugar kombucha solution at $p<0.05$. Black and green kombucha teas showed higher TPC compared to black and green teas at day 14 at $p<0.05$. Green kombucha tea has the highest TPC compared to all groups.

Table 3.4. The mean ± SEM of Total Phenolic Content (mg GAE) between groups

Groups	Mean (mg GAE) ±SEM		
	Day 0	Day 7	Day 14
Black Tea	11.72±1.23 [a]	15.56±0.70[a]	12.17±0.82 [a, c]
Green Tea	15.43±1.03[a]	15.54±0.76[a]	11.47±0.44 [a, c]
Sugar Solution	2.68±0.11	2.80±0.01	2.76±0.06
Black Kombucha Tea	15.92±1.01[a]	17.29±0.18[a]	16.50±0.97 [a, b]
Green Kombucha Tea	16.77±0.17[a]	17.82±0.21[a]	17.00±1.27 [a, b]
Sugar Kombucha Solution	3.53±0.02	3.81±0.20	3.83±0.05

a: significant compared to sugar solution and sugar kombucha solution at $p<0.05$
b: significant compared to green and black tea at $p<0.05$
c: significant compared to day 7

DISCUSSION

From the finding, the pH values of all groups were decreased or become more acidic may be due the fermentation process. According to Goh et al., (2012), a significant decreased in pH during the fermentation process is due to the conversion of glucose to gluconic acid. Moreover, the pH value also decreased due to an increase of organic acids formed during the fermentation process by bacteria and yeasts in the tea fungus combination (Jayabalan et al., 2008). The pH values of black kombucha tea, green kombucha tea and sugar kombucha solution were decreased minimally from day 7 to day 14. The possible mechanism is maybe the amphiprotic hydrocarbonate anion (HCO3-), which readily reacts with hydrogen ions (H+) from organic acids, may have been formed when the obtained water solution of carbon dioxide dissociated. This reaction prevents further changes in the H+ concentration and contributes to the system's buffering properties, thus lower the pH level (Jayabalan et al., 2008).

Different sample has a slightly different in the phytochemical compound. Similar finding as Malbaša et al., (2011), the researcher claimed that different chemical compositions of black and green tea may account for the differential in pH values between the substrates. In this study, phenol and flavonoid were detected in black tea, black kombucha tea, green tea and green kombucha tea. Meanwhile saponin was only present in black tea, black kombucha tea and green tea while tannin is detected in green tea, black tea and green kombucha tea. According to Montero et al., (2018), the flavonoid is well known antioxidative agents, hence its effects on human health are considerable. Flavonoid is a natural substance with polyphenolic structures and found abundant in plants (Panche et al., 2016). It is subcategorised mainly into flavones, flavonones, cathechins and anthocyanins and function of flavonoids are best portrayed as powerful antioxidant especially from flavones and cathechins (Nijveldt et al., 2001). Flavonoids also have been linked to a few clinical health benefits such as anti-atherosclerosis, anti-viral, anti-tumour and anti-inflammatory effect (Suyun et al., 2015).

Phenols are natural antioxidants found in various plant-based food such as fruits andleaves of vegetables (Kumar & Goel, 2019). Phenols have a higher potential in antioxidant activity and inhibits oxidative damage induced disease like stroke (Kumar& Goel, 2019). A study conducted by Cheng et al., (2017), revealed polyphenol was able to decrease the generation of ROS by blocking oxidases, lowering superoxide levels, inhibiting platelet aggregation, and resolving mitochondrial oxidative stress. Furthermore, the researcher claimed that consuming polyphenol may improves risk of hyperlipidemia, hypertension, diabetes mellitus, and obesity.

Tannin is a water-soluble compound with polyphenolic structure and are usually classified into hydrolysable and condensed tannins (Sieniawska et al., 2017). It is an essential plant constituents because of their scavenging ability (Value et al., 2012). Tannins are found nutritionally abundant in medicinal herbs, cereal, fruits like bananaand tea as well as in parts of the plants, such as leaves and flowers (Huang et al., 2018). The pharmacological activity of tannin such as antimicrobial (Cho et al., 2008), anti-tumour and anti-carcinogenic activity have been found mainly due to Type A tannins such as ellagitannins (Cristina et al., 2014). Study reported that tannins have direct effects on microorganism metabolism by inhibiting oxidative phosphorylation and inhibiting enzyme activity by complexing with substrates of bacteria and fungi (Wafa et al., 2016).

Saponin is another phytochemical compound found in the kombucha tea. This compound reported to show several antioxidative mechanism by acting as a primary antioxidant, that has the proton-donating ability and inhibits free radical production

(Akinpelu et al., 2014). By preventing the generation of oxygen free radicals, saponin helps to maintain the physicochemical characteristics of the membrane bilayer from free radical-induced cellular dysfunction. Moreover, study found that saponins yields different biological properties based on their plant type (Hussain et al., 2019). Saponins have been reported to exert health benefits such as reducing risk of hypercholesterolemia and cancer, and lower blood glucose level (Shi et al., 2004). The phytochemical screening may support the finding of antioxidant activities in kombucha tea. From the study, black kombucha tea, green kombucha tea and sugar kombucha solution showed high DPPH radical scavenging activity on day 14 of fermentation. It is believed that presence polyphenols, flavonoids and catechins are primarily responsible for their antioxidative effects. Furthermore, phenolic compounds could readily donate hydroxyl hydrogen due to resonance stabilization (Jayabalan et al., 2008). Jayabalan et al., (2014) reported that the presence of tea polyphenols and ascorbic enhances the the antioxidant activity of kombucha tea. In comparison with unfermented tea, kombucha tea found to have low molecular weight components and structural changes of tea polyphenols by enzymes produced by bacteria and yeast during fermentation. Moreover, microbial hydrolysis in the fermented tea increases the amount of phenolic and flavonoids compound, thus this increased the antioxidant activity of the beverage (Kim et al., 2014). Green kombucha tea has the highest DPPH radical scavenging activity among all groups. This can be described that green tea had the highest ability as hydrogen-donating, followed by black tea (Atoui et al., 2005). Green tea found to be rich in numerous compounds and metabolites such as tocopherols, polyphenols, ascorbic acids, carotenoids and catechins, hence it can be expressed as a sourceful antioxidant beverage. These compounds could escalate the green tea polyphenols antioxidant ability (Cabrera et al., 2006).

Similar to scavenging activity, black kombucha tea and green kombucha tea have the higher TPC among groups. This may be the result of kombucha's acidic environment due to the enzymes liberated by bacteria and yeast in tea fungus consortium (Jayabalan et al., 2008). This acidic condition retain the stability of flavonoids, proanthocyanidins and flavin. Srihari & Satyanarayana, (2012), reported that the degradation of epicatechin isomers occurs during kombucha fermentation. Moreover, enzymes produce by the bacteria and yeast in the fermented tea, break down the complex polyphenol structures into smaller molecules, results in the increase of TPC level.

From the study, the duration of fermentation, type of teas used and presence of kombucha culture flora influence the strength of the oxidation activity. Even though the oxidation properties of kombucha demonstrated time-dependent profiles, harmful effect may be arisen in prolong fermentation process due to accumulation of organics acids.

CONCLUSION

Fermented black and green kombucha tea decreased in pH after 14 days of fermentation. Moreover, both kombucha tea showed almost similar phytochemical compound presence in teas solution. The higher antioxidant activities were observed in both kombucha tea based on DPPH radical scavenging activity and TPC after 14 days of fermentation compared to black and green tea. Presence of phytochemical compound such flavonoids, phenol, saponin and tannin which may be responsible for their antioxidant activity.

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