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Combinations of traditional kombucha tea with medicinal plant extracts for enhancement of beneficial substances and activation of apoptosis signaling pathways in colorectal cancer cells

Thida KAEWKOD[1], Sirikwan SANGBOONRUANG[2], Supakit KHACHA-ANANDA[3], Sonthirat CHAROENRAK[1], Sakunnee BOVONSOMBUT[1], Yingmanee TRAGOOLPUA[1]*

Abstract

Traditional kombucha tea was prepared by black tea leaves before fermentation with symbiotic microorganisms for 15 days. In this study, kombucha was prepared through a combination with medicinal plant extracts of *T. catappa* (KT) and *A. marmelos* (KA) to enhance a number of beneficial aspects. The results revealed that the phenolics, flavonoids, antioxidants and six organic acids, namely glucuronic, gluconic, D-saccharic acid 1,4-lactone (DSL), acetic, ascorbic, and succinic acids, in kombucha combined with the medicinal plant extracts resulted in the production of substances with greater beneficial properties than traditional kombucha tea. The viability of colorectal cancer cells (Caco-2) after treatment with KT and KA was suppressed in a dose-dependent manner, while DNA fragmentation in Caco-2 cells was induced via the apoptosis mechanism. This process involved the apoptosis pathways related to the intrinsic apoptosis pathway, which was activated by KT and KA through the mitochondrial-dependent pathways including cytochrome c release and Bcl-2 suppression, and activation of caspases-9 and caspases-3. The findings of this study support the enhanced beneficial properties of traditional kombucha tea through a combination with medicinal plants. This outcome would also support the consideration of natural supplementary kombucha beverages as medicinal food products in the prevention of colorectal cancer.

Keywords: anticancer activity; apoptosis; beneficial substances; medicinal plant extract; traditional kombucha tea.

Practical Application: Combination of kombucha with *T. catappa* and *A. marmelos* showed increment of beneficial substances.

1 Introduction

Traditional kombucha tea is brewed with the use of black tea leaves and sugar combined with a symbiotic association of bacteria and yeasts to form a tea fungus. Kombucha tea originated in northeast China (Manchuria) and was known as red tea. It was so named because kombucha tea took on the color of the fermented and brewed liquid rather than the color of the black tea leaves. Kombucha tea has been known for its detoxifying and energizing properties. Moreover, kombucha tea was initially consumed in East Asia for its healing benefits to the body such as treatment of digestive problems. Consequently, the phrase 'kombucha tea' was internationally accepted as a fermented tea beverage and became fairly routine drinking fermented tea throughout Europe (Sandor, 2012; Goldstein, 2015). Additionally, the popularity of kombucha increased dramatically and it was suggested as a beneficial fermented food that is similar to those associated with the consumption of yogurt (Dufresne & Farnworth, 2000). To date, kombucha tea has become popular worldwide and is sold in a wide range of flavors.

Fermentation of kombucha tea involves metabolic activities between yeasts and acetic acid bacteria that utilize substrates in different ways (Teoh et al., 2004; Chakravorty et al., 2016; Jafari et al., 2021). The metabolic relationship of these microorganisms during fermentation produces beneficial organic acids and other substances such as acetic, gluconic acid, glucuronic acid, citric acid, lactic acid, malic acid, succinic acid, saccharic acid, pyruvic acid, sugars, vitamins, and amino acids (Jayabalan et al., 2014; Lee et al., 2021). Furthermore, the antioxidant activities, and phenolic and flavonoid compounds, are attributed to the polyphenols that exist in the tea leaves (Williamson et al., 2005). Black tea leaves are used as the general substrate in preparing traditional kombucha tea. The tea leaves contribute to the good flavor and taste of kombucha tea. However, organic acids, antioxidant activity, and other substances exhibit a number of beneficial effects that should be further studied in the hopes of enhancing the beneficial properties of traditional kombucha tea. This can be achieved through supplementation with medicinal plants.

Medicinal plants are a significant source of therapeutic phytochemicals that can contribute to the development of novel drugs. Most phytochemical compounds derived from medicinal

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[1]*Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand*
[2]*Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand*
[3]*Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
*Corresponding author: yboony150@gmail.com; yingmanee.t@cmu.ac.th

plants, such as phenolic and flavonoids groups, are reported to possess a range of capabilities in the treatment of diseases and in the prevention of cancer. The high content of phenolics and flavonoids found in medicinal plants are associated with their antioxidant activities, while their function contributes to the prevention of a number of diseases (Venugopal & Liu, 2012). In this study, the leaves of *Terminalia catappa* L. and the fruit of *Aegle marmelos* (L.) Correa ex Roxb., which have been used as traditional forms of medicine in Thailand, were investigated in terms of their anti-cancer activities (Subhadrabandhu, 2001). Over time, the leaves of *T. catappa* have been used to treat a number of diseases such as diarrhea, leprosy, coughs, jaundice, indigestion, headaches, colic, and swollen rheumatic joints (Anand et al., 2015). Likewise, *A. marmelos* is a popular fruit juice in Thailand that demonstrates a therapeutic potential in the treatment of many diseases such as chronic diarrhea, peptic ulcers, and respiratory infection. Additionally, these medicinal plants have been reported to be rich in antioxidant activities and phytochemical substances (Chitmanat et al., 2005; Baliga et al., 2011). Moreover, kombucha tea supplement with black carrot has been studied by Yildiz et al. (2021) and revealed the increment of antioxidant capacities such as anthocyanin and phenolic compounds. However, in-depth studies on traditional kombucha tea in combination with medicinal plants have not yet been conducted. Consequently, the full recognition of these beneficial substances has not been recognized in terms of their potential anticancer activities. Thus, this study aimed to evaluate the effects of traditional kombucha tea prepared from black tea (KB) in combination with the medicinal plant extracts of *T. catappa* (KT) and *A. marmelos* (KA). Ultimately, we hope to promote the potentially beneficial properties of these substances through the enhancement of their total phenolic and flavonoid contents and their antioxidant activities will be assessed. Moreover, in this study, the potential anti-colorectal cancer (CRC) activities that are related to the induction of apoptosis mechanisms established through a combination of kombucha with medicinal plant extracts will also be investigated and compared to traditional kombucha tea.

2 Materials and methods

2.1 Preparation of traditional kombucha tea

Traditional kombucha tea was prepared by combining black tea at 1.0% w/v with sucrose at 10% w/v. The tea leaves were added to sterilized water and then boiled for 15 min. Sucrose was dissolved in hot tea and the tea was then filtered into sterilized glass bottles. Starter culture comprised of tea fungus at 10% v/v was inoculated into the tea broth and the tea was further incubated at room temperature for 15 days. Moreover, kombucha tea samples were sterilized by being filtered through a sterile microfilter (0.22 μm pore size) and kept at -20 °C. Black tea leaves and starter culture were kindly obtained from the Tea Gallery Group (Thailand) Co., Ltd., Chiang Mai, Thailand.

2.2 Medicinal plant collection and sample extraction

The *Aegle marmelos* (L.) Correa ex Roxb. (Voucher No. WP7681) fruits were purchased from Waroros Market, Chiang Mai, Thailand, in May 2014. The fresh *Terminalia catappa* L. (Voucher No. WP7682) leaves were collected from Chiang Mai University, Chiang Mai, Thailand, in October 2014. All plants were indentified and authenticated by a botanist, Asst. Prof. Dr. Angkhana Inta, at the Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. The plants were dried and ground in order to achieve extraction by soaking them in hot distilled water at a ratio of 1:10 w/v for 1 h. The extraction process was then repeated 3 times (Chan et al., 2011). The resulting extract was filtered using Whatman's No.1 filter paper. Subsequently, the filtrate of the extract was evaporated using a rotary evaporator at 45 °C under reduced pressure of 50 mbar to remove any solvents. Plant extracts were then dried by lyophilization and kept at -20 °C. The resulting plant extracts were dissolved in sterile distilled water at a concentration of 500 mg/mL and kept at -20 °C until being used.

2.3 Combination of kombucha with medicinal plant extracts

Kombucha prepared from black tea (100%) after 15 days of fermentation was combined with each medicinal plant extract (500 mg/mL) in order to produce kombucha with *T. catappa* (KT) and kombucha with *A. marmelos* extract (KA). The efficacy of kombucha combined with these two medicinal plant extracts was studied in terms of the anticancer activities of these preparations and the potential applications of their beneficial substances.

2.4 Determination of beneficial substances in kombucha tea combined with medicinal plant extracts

The pH of kombucha tea combined with medicinal plant extracts was measured using an electronic pH meter (Denver Instruments, Bohemia, NY, USA). Moreover, the predominant organic acids present in kombucha tea combined with the medicinal plant extracts were determined using high performance liquid chromatography (HPLC). Organic acids present in the samples were detected using isocratic HPLC systems with a conventional C18 column and compared to standard organic acids including glucuronic acid (Sigma-Aldich, Darmstadt, Germany), gluconic acid (Merck, Darmstadt, Germany), D-saccharic acid 1,4-lactone (DSL) (Sigma-Aldich, Germany), acetic acid (Merck, Germany), ascorbic acid (Merck, Germany), and succinic acid (Merck, Germany). The samples were filtered through a 0.22 μm sterile microfilter and 50 μL of the filtrate was injected into the HPLC system (Agilent Technologies 1,200 series, Santa Clara, CA, USA). A C-18 column (4.6 × 150 mm, 5 μm; GL Sciences, Tokyo, Japan) employing a UV detector (210 nm) was used for the analysis. Six organic acids present in the kombucha combined with the medicinal plants were separated with 20 mM KH_2PO_4 and pH 2.4 elution buffer. Furthermore, the HPLC system was maintained at a flow rate of 0.8 mL per minute and a running time of 40 min at 25 °C (Kaewkod et al., 2019). The peak area of the standard organic acids was calculated with the use of the Agilent Chem Station level-5 program and then standard graphs of each organic acid were generated. Subsequently, the content of each organic acid in the sample was calculated from the standard graph of each organic acid.

2.5 Antioxidant activity of kombucha combined with medicinal plant extracts

The radical scavenging activity of kombucha tea combined with medicinal plant extracts was determined against DPPH free radicals (Miliauskas et al., 2004). The samples were 10-fold serial diluted with methanol. Each concentration of the samples (0.5 mL) was incubated with 1.5 mL of 0.1 mM DPPH solution (Sigma-Aldich, Germany). Absorbance at 517 nm was measured with the use of a spectrophotometer (Genesys 20, Thermo Scientific, Dreieich, Germany) after the solution mixture was kept in the dark at room temperature for 20 min. Methanol was used as a blank solution and DPPH without a sample was used as a control. The percentage of DPPH free radical inhibition was calculated as follows (Equation 1):

$$Percentage\ inhibition = \left\{\left(A1 - A2\right)/A1\right\} x\ 100 \quad (1)$$

wherein, A1 represents the absorbance of the DPPH solution and A2 represents the absorbance of the sample with the DPPH solution. Antioxidant activity of the samples was demonstrated by comparing standard gallic acid and expressed as milligrams of gallic acid equivalent per milliliter of the sample (mg GAE per mL kombucha).

2.6 Total phenolic compounds of kombucha combined with medicinal plant extracts

The total phenolic contents of kombucha tea combined with medicinal plant extracts were determined using the Folin-Ciocalteu method (Singhatong et al., 2010). Each sample (250 µL) was mixed with 125 µL of 50% Folin-Ciocalteu reagent (Merck, Germany) and 250 µL of 95% ethanol. The mixture was then incubated in the dark at room temperature for 5 min. Subsequently, 250 µL of 5% sodium carbonate was added and the mixture was incubated in the dark at room temperature for 60 min. The blue molybdenum-tungsten complex was formed and detected at 725 nm using a spectrophotometer (Genesys 20, Thermo Scientific, Germany). The total phenolic content was calculated by comparing it to the standard gallic acid equivalent. It was then expressed as milligrams of gallic acid per milliliter of the sample (mg GAE per mL kombucha).

2.7 Total flavonoid content of kombucha combined with medicinal plant extracts

The total flavonoid contents of kombucha tea combined with medicinal plant extracts were determined using the aluminum chloride colorimetric method (Singhatong et al., 2010). Samples (500 µL) were mixed with 100 µL of 10% aluminium chloride, 1.5 mL of methanol, 100 µL of 1 M potassium acetate, and 2.80 mL of deionized water. The mixture was then incubated in the dark at room temperature for 30 min. The solution value was then detected at 415 nm using a spectrophotometer (Genesys 20, Thermo Scientific, Germany). Total flavonoid content was calculated by comparing it to the standard quercetin equivalent and expressed as milligrams of quercetin per milliliter of the sample (mg QUE per mL kombucha).

2.8 Cultivation of cancer cells

Human colorectal cancer cells (Caco-2) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyCloneTM, Pittsburgh, PA, USA), 100 Units/mL penicillin, and 100 µg/mL streptomycin (CAISSON, Smithfield, UT, USA). After being incubated at 37 °C in a 5% CO_2 incubator (SHEL LAB, Cornelius, OR, USA), the cells were washed twice with phosphate buffer saline (PBS, pH 7.4) and trypsinized with 0.05% trypsin-EDTA solution (CAISSON, USA). The cells were then split at a ratio of 1:3 and incubated at 37 °C in a 5% CO_2 incubator until confluence was achieved. Cells were then harvested after trypsinization with 0.05% trypsin-EDTA solution in a sterile centrifuge tube. The pellet cells were centrifuged at 1,200 g at a temperature of 4 °C for 5 min. Ultimately, the cells were resuspended with the appropriate medium and counted with a hemacytometer in order to determine the number of cells.

2.9 Cytotoxicity of kombucha tea combined with medicinal plant extracts on cancer cells

The cytotoxicity of kombucha tea combined with medicinal plant extracts was tested using MTT assay (Umthong et al., 2011). Caco-2 human colorectal carcinoma cells were plated into 96-well plates and incubated at 37 °C in a 5% CO_2 incubator for 24 h. After incubation, either kombucha tea or the medicinal plant extract was added. The plates were then incubated at 37 °C in a 5% CO_2 incubator for 48 h. MTT solution (Bio Basic Inc., Amherst, NY, USA) was then added to the specimens and they were incubated for 4 h. Finally, blue formazan crystals were dissolved with dimethyl sulfoxide (DMSO) and the degrees of absorbance of the solutions were measured at 540 and 630 nm using a microplate reader (EZ Read 2000, Biochrom, Cambridge, UK). The percentage of cell viability was calculated and compared to the cell control.

2.10 DNA fragmentation

Caco-2 human colorectal carcinoma cells (2×10^5 cells per mL) were cultivated in 24-well plates and incubated at 37°C in a 5% CO_2 incubator for 24 h. Kombucha combined with medicinal plant extracts was added and the cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Cells were harvested after being trypsinized with 0.05% trypsin-EDTA solution and washed three times with phosphate buffer saline (PBS, pH 7.4). Pellet cells were lysed with 30 µL of lysis solution (10 mM Tris-HCl, 2.5 mM EDTA, 100 mM NaCl, 1% SDS, pH 8.0) and the solution was mixed with a vortex mixer. Then, cold 5 M NaCl, 10 mg/mL Proteinase K, and 10 mg/mL RNaseA were added and the solution was further mixed using the vortex mixer. The solution was further incubated at 37 °C for 3 h. After incubation, DNA fragmentation was detected on 2% agarose gel at 60 volts for 3 h and visualized by ultraviolet transilluminator (Zar et al., 2014).

2.11 TUNEL assay of kombucha tea combined with medicinal plant extracts on cancer cells

Detection of DNA damage on cancer cells after treatment with kombucha combined with medicinal plant extracts was

investigated using TUNEL assay (DNA Fragmentation Imaging kit, Merck, Germany). Caco-2 human colorectal carcinoma cells (2×10^5 cell per mL) were treated with the samples for 24 h. Cells were harvested and washed three times with phosphate buffer saline (PBS, pH 7.4). Cells were then fixed with 100 µL of 4% paraformaldehyde and incubated at room temperature for 10 min. After removing the fixing solution, 100 µL of 0.1% Triton-X 100 was added and cells were incubated at room temperature for 20 min. Cells were then washed twice with phosphate buffer saline (PBS, pH 7.4). After centrifugation, the cells were mixed with 45 µL of the enzyme solution (Terminal deoxynucleotidyl transferase, TdT) and incubated at 37 °C for 1 h. Then, 150 µL of nuclei dye mixture solution (Hoechst 33342) was added and the cells were incubated in the dark at room temperature for 15 min. The reagent was removed by centrifugation at 5,000 g and 4 °C for 5 min. Pellets were resuspended with ProLongTM gold antifade mountant (Life technologies, Camarillo, CA, USA) before detection by inverted fluorescence microscope (ECLIPSE Ts2R-FL, Nikon, Tokyo, Japan).

2.12 Determination of apoptotic gene expression in cancer cells by quantitative real-time polymerase chain reaction (qRT-PCR)

The mRNA of apoptotic gene expression, including *Bcl-2*, *cytochrome c*, and *caspase-8*, were investigated after treatment with kombucha combined with medicinal plant extracts by applying qRT-PCR. Caco-2 human colorectal carcinoma cells (2×10^5 cell per mL) were treated with kombucha combined with medicinal plant extracts for 1 h. Cells were then harvested and washed three times with phosphate buffer saline (PBS, pH 7.4) before RNA extraction. Total mRNA of Caco-2 cells was extracted using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) and reversed to cDNA using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan). The master mix of the reaction contained 100 ng of cDNA, 2X SensiFAST SYBR® No-ROX Mix (BIOLINE, London, UK), 400 nM of the forward primer, and 400 nM of the reverse primer. PCR conditions were applied as follows. Denaturation was performed at 95 °C for 2 min. Amplification and quantification were repeated for 40 cycles at 95 °C for 5 secs and 60 °C for 30 secs. Cycle threshold (C_t) values of the apoptotic genes were calculated from the relative expression by normalizing the data with the expression of the internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the forward and reverse primers for PCR were designed based on previous studies. Primer sequences for the amplification of apoptotic genes are shown in Table 1.

2.13 Determination of apoptotic protein expressions in cancer cells using SDS-PAGE and Western blotting analysis

The ability of kombucha tea combined with medicinal plant extracts to induce the apoptotic protein expression of Caco-2 human colorectal carcinoma cell was assessed through the expression of Bcl-2, cytochrome c, caspase-9, and caspase-3. Caco-2 cells (2×10^5 cell per mL) were cultivated in 24-well plates and then incubated at 37 °C in a 5% CO_2 incubator for 1 h. After that, kombucha combined with medicinal plant extracts was added and the cells were incubated at 37 °C in a 5% CO_2 incubator for 1 h. The cells were harvested and washed three times with phosphate buffer saline (PBS, pH 7.4). After the removal of PBS, cells were lysed with RIPA buffer in a protease inhibitor cocktail (1:100) on ice for 60 min and were then mixed every 10 min with a vortex mixer. The debris cells were removed by centrifugation at 3,000 g and 4°C for 15 min. The supernatant was then collected and the amount of protein was calculated using a BCA assay kit (Merck, Germany). Thereafter, the apoptosis protein of cytochrome c was extracted using a Mitochondria/Cytosol Fraction Kit (BioVision, Milpitas, CA, USA) and the amounts of protein in the mitochondria and cytosol fractions were determined by Bradford assay. Thirty micrograms of protein from each sample were loaded on polyacrylamide gel (15%) and the separating gel was transferred into the nitrocellulose membrane using western blotting. The membrane was probed with specific primary antibodies (Merck, Darmstadt, Germany), namely mouse anti-cytochrome c (1:400), rabbit anti-Bcl-2 (1:1,000), mouse anti-caspase-9 (1:1,000), mouse anti-caspase-3 (1:500), and rabbit anti-GAPDH (1:3,000), and then incubated overnight at 4°C. The membrane was then probed with 1:1,000 anti-mouse or anti-rabbit conjugated with horseradish peroxidase (Merck, Darmstadt, Germany). After 1 hour of incubation, 500 µL of chemiluminescent western ECL (Immobilon® Forte Western HRP Substrate, Merck, Darmstadt, Germany) was spread on the membrane and it was left at room temperature for 3-5 min before being exposed to clear blue X-Ray film (CL-XPosure Film, Thermo Scientific, Waltham, MA, USA).

2.14 Statistical analysis

All investigations were performed by way of three independent experiments on separate occasions. All data were presented as mean ± SD values of the independent sample. T-test and ANOVA analysis were used to analyze results of both the treatment and control groups.

Table 1. Oligonucleotide primers used for qRT-PCR.

Genes	Sequence of PCR primers (5'–3')	References
Bcl-2	Forward: GTCTGGGAATCGATCTGGAAATCC Reverse: TTTGAAACTTCCCAATGAATCAGGAG	Kim et al. (2011)
cytochrome c	Forward: GAGCGGGAGTGTTCGTTGT Reverse: GTCTGCCCTTTCTTCCTTCT	Sun et al. (2012)
caspase-8	Forward: GTGGAGGAAAGCAATCTGTC Reverse: TATTAGCCCTGCCTGGTGTCT	Ge et al. (2011)
GAPDH	Forward: GAAGGTGAAGGTCGGAGTC Reverse: GAAGATGGTGATGGGATTTC	Sun et al. (2012)

3 Results

3.1 Physical properties and organic acid contents of kombucha combined tea with medicinal plant extracts

Kombucha combined with *Terminalia catappa* (KT) and kombucha combined with *Aegle marmelos* extract (KA) were analyzed as beneficial substances and compared to traditional kombucha prepared from black tea (KB). Both the KB and KT kombucha mixtures appeared as brown in color, while KA mixture appeared as yellow-brown in color. The pH value of kombucha tea prepared from black tea was 2.70, which was the lowest pH value of all forms of kombucha in this study. After the kombucha tea had been combined with *T. catappa* and *A. marmelos* extracts, pH values of 2.75 and 3.26 were recorded, respectively (Table 2). Subsequently, beneficial organic acids in KT and KA were analyzed by HPLC assay. The amounts of six organic acids, including glucuronic acid, gluconic acid, D-saccharic acid 1,4-lactone (DSL), acetic acid, ascorbic acid, and succinic acid, were determined. The results revealed that the number of organic acids in the kombucha tea combined with plant extracts; KT and KA, increased when compared with the kombucha prepared without these plant extracts. Hence, extracts of *T. catappa* and *A. marmelos* were found to have enhanced the beneficial aspects associated with the organic acids present in the kombucha tea that was prepared from black tea (Table 2).

With regard to KT, the amount of all organic acids was found to be significantly higher than in the kombucha prepared from black tea. Furthermore, the highest content of gluconic acid at a value of 33.10 ± 2.64 g/L was found in KT, followed by ascorbic acid, DSL, succinic acid, glucuronic acid, and acetic acid (Table 2). In this study, the amounts of organic acids in the *T. catappa* extract were also determined. From assessment of the HPLC profile, all organic acids in the *T. catappa* extract (TE) were measured. DSL was found to contain the highest content of 228.65 ± 60.38 mg per g extract (Table 2). In addition, KA contained significantly higher amounts of glucuronic acid, DSL, acetic acid, and succinic acid than the kombucha that had been prepared from black tea. Additionally, KA reported the highest content of ascorbic acid at a value of 21.34 ± 0.86 g/L, followed by succinic acid, gluconic acid, acetic acid, glucuronic, and DSL (Table 2). Moreover, the four organic acids present in *A. marmelos* extract (AE) were glucuronic acid, gluconic acid, acetic acid, and succinic acid. Gluconic acid was detected in the highest amount at 38.76 ± 1.96 mg per g extract (Table 2). Furthermore, the amount of all organic acids in KT were higher than in KA. In contrast, the amount of acetic acid in KA was significantly higher than in KT (Table 2).

3.2 Antioxidant activity, total phenolic compounds and flavonoid content of kombucha combined with medicinal plant extracts

The radical scavenging activities of kombucha combined with *T. catappa* (KT) and kombucha combined with *A. marmelos* (KA) were determined against DPPH free radicals using DPPH radical scavenging assay. In addition, total phenolic compounds and flavonoid contents were also investigated in both KT and KA. After combining kombucha prepared from black tea with *T. catappa* or *A. marmelos* extracts, the antioxidant activity, total phenolic compounds and flavonoid contents were found to be significantly higher than in the kombucha prepared from black tea without the medicinal plant extracts. The antioxidant activity in KT was significantly higher than in KA since the modified kombucha tea could be scavenged for DPPH radicals at values of 7.23 ± 0.02 and 0.72 ± 0.00 mg GAE per mL kombucha, respectively. Furthermore, the antioxidant activity of kombucha prepared with black tea was 0.38 ± 0.02 mg GAE per mL kombucha (Table 3). Moreover, the highest antioxidant activity correlated to the amounts of the phenolic and flavonoid compounds. The highest amount of phenolic compounds was found in KT at 37.55 ± 1.78 mg GAE per mL kombucha, followed by KA at 23.15 ± 1.44 mg GAE per mL kombucha (Table 3). Moreover, KT was also found to possess the highest amount of flavonoids at a value of 4.19 ± 0.11 mg QUE per mL kombucha, followed by 0.49 ± 0.03 mg QUE per mL kombucha in KA (Table 3). In addition, antioxidant activity, and total phenolic and flavonoid compounds, were also recorded in *T. catappa* and *A. marmelos* extracts (Table 3).

3.3 Cytotoxicity of kombucha combined with medicinal plant extracts on Caco-2 cancer cells

The degrees of cytotoxicity of kombucha prepared from black tea (KB), kombucha combined with *T. catappa* (KT), and kombucha

Table 2. Physical properties and organic acid contents of kombucha combined with medicinal plant extracts.

Samples	Color	pH [1]	Organic acid contents in kombucha (g/L) and medicinal plant (mg per g extract) [1]					
			Glucuronic	Gluconic	DSL	Acetic	Ascorbic	Succinic
KB	Brown	2.70 ± 0.07	ND^{a}	13.57 ± 0.14^{a}	1.16 ± 0.18^{a}	0.15 ± 0.03^{a}	21.69 ± 0.39^{a}	0.89 ± 0.23^{a}
KT	Brown	2.75 ± 0.01	10.68 ± 2.59^{b*}	33.10 ± 2.64^{b*}	18.61 ± 1.08^{b*}	1.66 ± 0.15^{b}	31.88 ±3.24^{b*}	16.49 ± 0.40^{b*}
KA	Yellow brown	3.26 ± 0.02	5.54 ± 0.06^{b}	12.80 ± 0.35^{a}	3.46 ± 0.21^{b}	8.64 ± 0.24^{b*}	21.34 ± 0.86^{a}	12.98 ± 0.42^{b}
TE	Brown	-	23.08± 1.95	76.81 ± 4.79	228.65 ± 60.38	26.19 ± 1.66	48.43 ± 16.50	89.08 ± 11.54
AE	Yellow brown	-	5.29 ± 1.05	38.76 ± 1.96	ND	17.94 ± 0.67	ND	16.36 ± 1.06

[1]The results are presented as mean ± SD values of triplicate independent experiments. ^{a,b} The data of different superscript letters (a, b) show significantly different values of KT and KA when compared with KB ($P < 0.05$). *The statistical data show the highest value in each sample ($P < 0.05$). ND: Organic acid in the sample was not detected. Abbreviations are indicated as follows: kombucha prepared from black tea (KB), kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), *T. catappa* extract (TE), and *A. marmelos* extract (AE).

combined with *A. marmelos* (KA) on Caco-2 cell proliferation were observed after treatment at various concentrations of the samples for 48 h. The proliferation of Caco-2 cells was found to be suppressed by the KB, KT, and KA in a dose-dependent manner. Caco-2 cell viability in the presence of KB (0.781% -12.5%) ranged from 3.51 ± 0.45 to 98.68 ± 3.56% (Figure 1A). After incubation of Caco-2 cells with KT (0.0975% - 1.56%), Caco-2 cell viability ranged from 6.62 ± 0.95 to 81.08 ± 3.63%. In addition, Caco-2 cell viability after treatment with KA (3.125% - 50%) ranged from 2.66 ± 0.49 to 116.12 ± 6.22%, respectively (Figure 1B and 1C). Moreover, after treatment of Caco-2 cells in each sample for 48 h, the morphology of the Caco-2 cells presents in the apoptosis cells in terms of cell shrinkage, membrane blebbing condensation, margination of nuclear chromatin, apoptotic bodies, and engulfment by neighbor cells were clearly observed when the concentration of each sample was increased (Figure 1D).

The 50% inhibitory concentration (IC_{50}) values of the KB, KT, and KA were calculated in order to compare the degrees of toxicity of each sample after treatment on Caco-2 cells. After combination of kombucha prepared from black tea combined with the plant extracts, KT exhibited significantly higher inhibitory activity against Caco-2 cells than KA and KB since the IC_{50} values of KT, KA and KB were 0.346 ± 0.008%, 13.704 ± 1.336%, and 6.077 ± 0.222%, respectively (Table 4). Moreover, MTT assay demonstrated that both plant extracts could also inhibit Caco-2 cells (Table 4). The extract of *T. catappa* presented the highest activity against Caco-2 cells with 50% inhibitory concentration (IC_{50}) of 0.169 ± 0.010 mg/mL. Additionally, *A. marmelos* extract displayed toxicity against Caco-2 cells at IC_{50} of 39.342 ± 8.773 mg/mL (Table 4).

3.4 Effect of kombucha combined with medicinal plant extracts on DNA fragmentation of Caco-2 cancer cells

Kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), and kombucha prepared from black tea (KB) were studied in terms of the apoptosis mechanisms by induction of DNA fragmentation on Caco-2 cells.

Table 3. Antioxidant activity, along with total phenolic and flavonoid contents of kombucha combined with medicinal plant extracts.

Samples	Antioxidant activity, total phenolic and flavonoid contents [1]		
	Antioxidant activity (mg GAE per mL kombucha or mg GAE per g extract)	Total phenolic content (mg GAE per mL kombucha or mg GAE per g extract)	Total flavonoid content (mg QUE per mL kombucha or mg QUE per g extract)
KB	0.38 ± 0.02[a]	0.35 ± 0.01[a]	0.02 ± 0.01[a]
KT	7.23 ± 0.02[b*]	37.55 ± 1.78[b*]	4.19 ± 0.11[b*]
KA	0.72 ± 0.00[b]	23.15 ± 1.44[b]	0.49 ± 0.03[b]
TE	100.77 ± 1.28	56.01 ± 0.19	29.66 ± 0.50
AE	7.10 ± 0.09	5.91 ± 0.05	3.01 ± 0.21

[1]The results are presented as mean ± SD values of triplicate independent experiments. Abbreviations are indicated as follows: kombucha prepared from black tea (KB), kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), *T. catappa* extract (TE) and *A. marmelos* extract (AE). [a,b]The data of different superscript letters (a, b) show significantly different values of KT and KA when compared with KB ($P < 0.05$). *The statistical data show the highest value in each sample ($P < 0.05$).

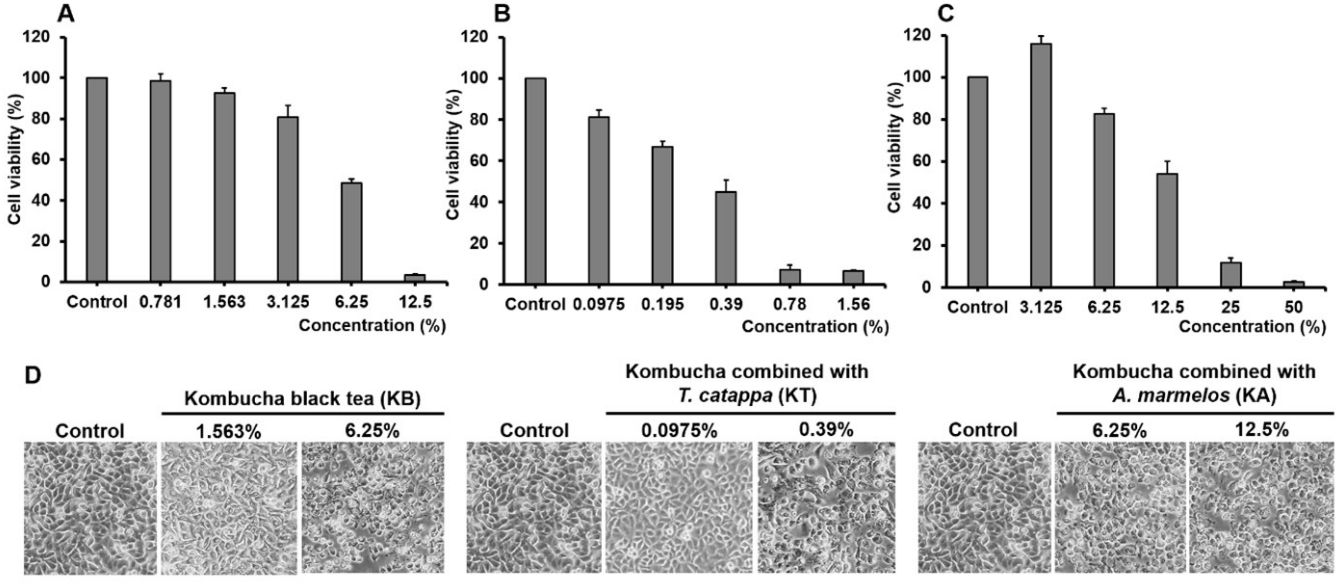

Figure 1. The cytotoxicity (A-C) and morphology appearance (D) of Caco-2 cells after treatment with kombucha prepared from black tea (KB); A, kombucha combined with *T. catappa* (KT); B and kombucha combined with *A. marmelos* (KA); C for 48 h. Cell viability was measured using the MTT assay and calculated for comparisons with the control cells. The results are presented as mean ± SD values of triplicate independent experiments.

Table 4. Values pertaining to the 50% inhibitory concentration (IC_{50}) of kombucha combined with medicinal plant extracts after treatment of caco-2 cells for 48 h.

Samples	50% inhibitory concentration (IC_{50}) of kombucha (%) and medicinal plants (mg/mL) [1]
KB	6.077 ± 0.222[a]
KT	0.346 ± 0.008[b*]
KA	13.704 ± 1.336[b]
TE	0.169 ± 0.010
AE	39.342 ± 8.773

[1]The results are presented as mean ± SD values of triplicate independent experiments. Abbreviations are indicated as follows: kombucha prepared from black tea (KB), kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), *T. catappa* extract (TE), and *A. marmelos* extract (AE). [a,b] The data of different superscript letters (a, b) reflect significantly different inhibitory concentration values of KT and KA when compared with KB ($P < 0.05$). *The highest potential inhibition of the sample to suppress Caco-2 cells is indicative of a significant value ($P < 0.05$).

Detection of DNA fragmentation in relation to the apoptosis in Caco-2 cells was performed after treatment with the samples at different concentrations. After treatment of Caco-2 cells with KT at concentrations of 0.175% to 0.70%, and KA at concentrations of 7.0% to 28.0% for 24 h, DNA fragments within a range of 100 to 500 bp were observed by agarose gel electrophoresis (Figure 1S). Moreover, kombucha prepared from black tea at concentrations of 3.0% to 12.0% induced the DNA fragmentation of Caco-2 cells. Moreover, *T. catappa* extract (0.1 mg/mL to 0.4 mg/mL) and *A. marmelos* extract (3.6 mg/mL and 14.4 mg/mL) also induced the DNA fragmentation of Caco-2 cells (Figure 1S).

3.5 Effects of kombucha combined with medicinal plant extracts on DNA damage of Caco-2 cancer cells

Detection of DNA damage on Caco-2 cells after treatment with kombucha combined with *T. catappa* (KT) and kombucha combined with *A. marmelos* (KA) was investigated using TUNEL assay. Additionally, kombucha prepared from black tea (KB) was also studied in terms of the above-mentioned parameters. All samples were found to be able to induce the DNA fragmentation of Caco-2 cells. In this study, the effect of kombucha and plant extracts on the DNA damage of Caco-2 cells was detected through staining with fluorescent dyes (Hoechst 33342 and TUNEL). Observations were made under a fluorescent microscope after treatment for 24 h. In the untreated cell control, Caco-2 cells were stained with only Hoechst 33342 fluorescence in the nucleus of cells. In contrast, TUNEL green fluorescence was detected in the cells to indicate DNA damage during the late stage of apoptosis. The results showed that the nucleus of the Caco-2 cell appeared as a green fluorescence from TUNEL staining after treatment with KT at a concentration of 0.35% and KA at a concentration of 14.0% (Figure 2). Similarly, kombucha prepared from black tea (KB) at a concentration of 6.0% also induced the DNA damage of Caco-2 cells (Figure 2). In addition, *T. catappa* extract (0.2 mg/mL) and *A. marmelos* extract (7.2 mg/mL) were also found to have induced the DNA damage of Caco-2 cells (Figure 2). These results were concordant with the effects of kombucha combined with medicinal plant extracts on the DNA fragmentation of Caco-2 cancer cells by agarose gel electrophoresis detection.

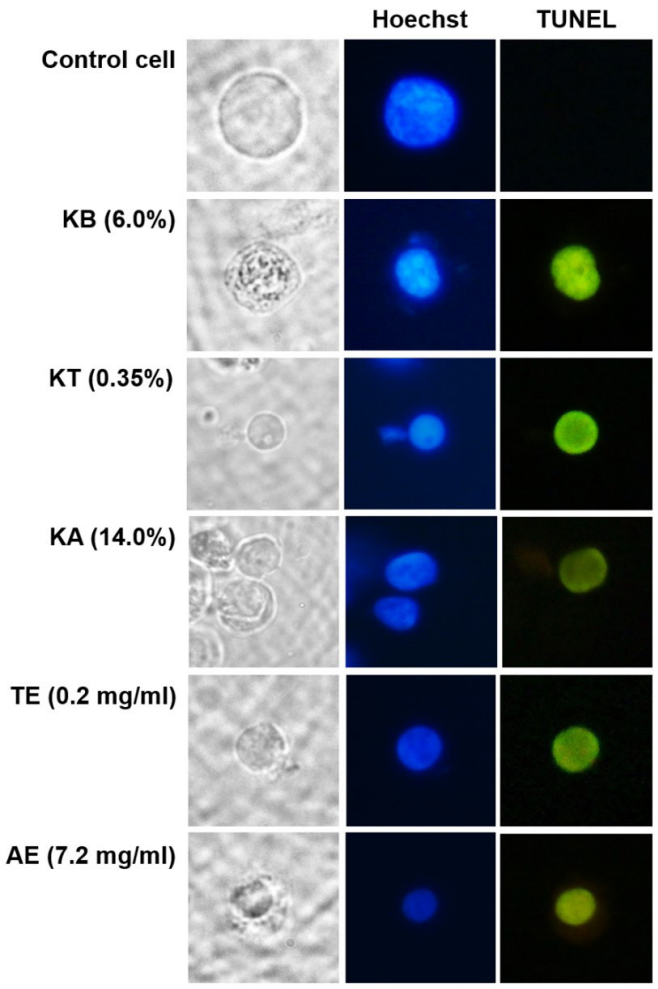

Figure 2. TUNEL assay of Caco-2 cells after treatment with kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), kombucha prepared from black tea (KB), *T. catappa* extract (TE) and *A. marmelos* extract (AE) when compared with control cells for 24 h. The cells were stained with TUNEL and Hoechst 33342, and observed under a fluorescent microscope.

3.6 Effects of kombucha combined with medicinal plant extracts on apoptotic gene expression of Caco-2 cancer cells

The apoptotic genes, namely *Bcl-2*, *cytochrome c*, and *caspase-8*, were investigated after Caco-2 cells were treated with kombucha combined with *T. catappa* (KT) and kombucha combined with *A. marmelos* (KA) by qRT-PCR. Additionally, kombucha prepared from black tea (KB) and kombucha combined with *T. catappa* and *A. marmelos* extracts were also studied. An increase in *cytochrome c* gene expression was found to be related to the intrinsic apoptosis pathway and the *Bcl-2* gene related to the apoptotic inhibitor of the intrinsic apoptosis pathway. In contrast, an increase in *caspase-8* gene expression was determined to be related to the extrinsic apoptosis pathway. The qRT-PCR indicated that the relative gene expression of *cytochrome c* significantly

revealed an upregulation after Caco-2 cells treated with KB (12.0%) and *A. marmelos* extract (7.2 mg/mL) when compared with the untreated cells (Figure 3). Therefore, all extracts were found to significantly downregulate the relative gene expression of *Bcl-2* after treatment with KT (0.35% and 0.70%), KA (7.0% and 14.0%), KB (6.0% and 12.0%), *T. catappa* extract (0.7 mg/mL and 1.4 mg/mL), and *A. marmelos* extract (3.6 mg/mL and 7.2 mg/mL) for 1 h (Figure 3). Moreover, the relative gene expression of *caspase-8* after all treatments was not determined to be different from that of the untreated control cells (Figure 3). These results indicated that all treatments were related to the intrinsic apoptosis pathway through the transcriptional activity of the *Bcl-2* and *cytochrome c* genes.

3.7 Effects of kombucha combined with medicinal plant extracts on apoptotic protein expression of Caco-2 cancer cells

Apoptotic protein expression values in Caco-2 cells after treatment with kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), and kombucha prepared from black tea (KB) were also studied. The relevant candidates of apoptotic proteins, such as cytochrome c, Bcl-2, caspase-9, and caspase-3, along with the protein expression values were detected after incubation at each concentration of all treatments for 1 h. The proteins were analyzed using SDS-PAGE and western blotting analysis with specific anti-apoptotic proteins. The expression levels of each apoptotic proteins are presented in Figure 4. The expression of GAPDH as the internal protein control was not affected after incubation of the Caco-2 cells at each concentration. The results indicated that the expression level of cytochrome c in the cytosol of cells was higher than what was observed in the mitosol of the cells after all treatments (Figure 4A and 4B). Moreover, KT at a concentration of 0.7% and KA at a concentration of 14.0% were found to be able to decrease the level of Bcl-2 protein expression when compared with the untreated cells. Additionally, KB (12.0%) combined with the extracts of *T. catappa* (1.4 mg/mL) and *A. marmelos* (3.6 mg/mL and 7.2 mg/mL) also affected the Bcl-2 protein by decreasing the levels of protein expression (Figure 4A and 4C).

Therefore, the expression levels of caspase-9 and caspase-3 were similarly expressed after Caco-2 cells were treated with KT (0.7%), KB (12.0%), *T. catappa* (0.7 mg/mL and 1.4 mg/mL), and *A. marmelos* (3.6 mg/mL and 7.2 mg/mL) extracts and then compared to the untreated control cells. This result demonstrated that the extracts were capable of inducing the intrinsic apoptosis pathway through the mitochondria-dependent pathways by activation of the cytochrome c accumulated in the cytosol of the cells. After that, activation of the caspase cascade of caspase-9 and caspase-3 proteins subsequently induced the apoptosis of the cancer cells (Figure 4A, 4D and 4E).

4 Discussion

Enhancement of the beneficial aspects of traditional kombucha tea was evaluated by combining the tea with medicinal plants. In this study, traditional kombucha tea was prepared by fermenting black tea with a symbiosis culture of acetic acid bacteria including *Acetobacter xylinum* and yeast cells for 15 days. In addition, our

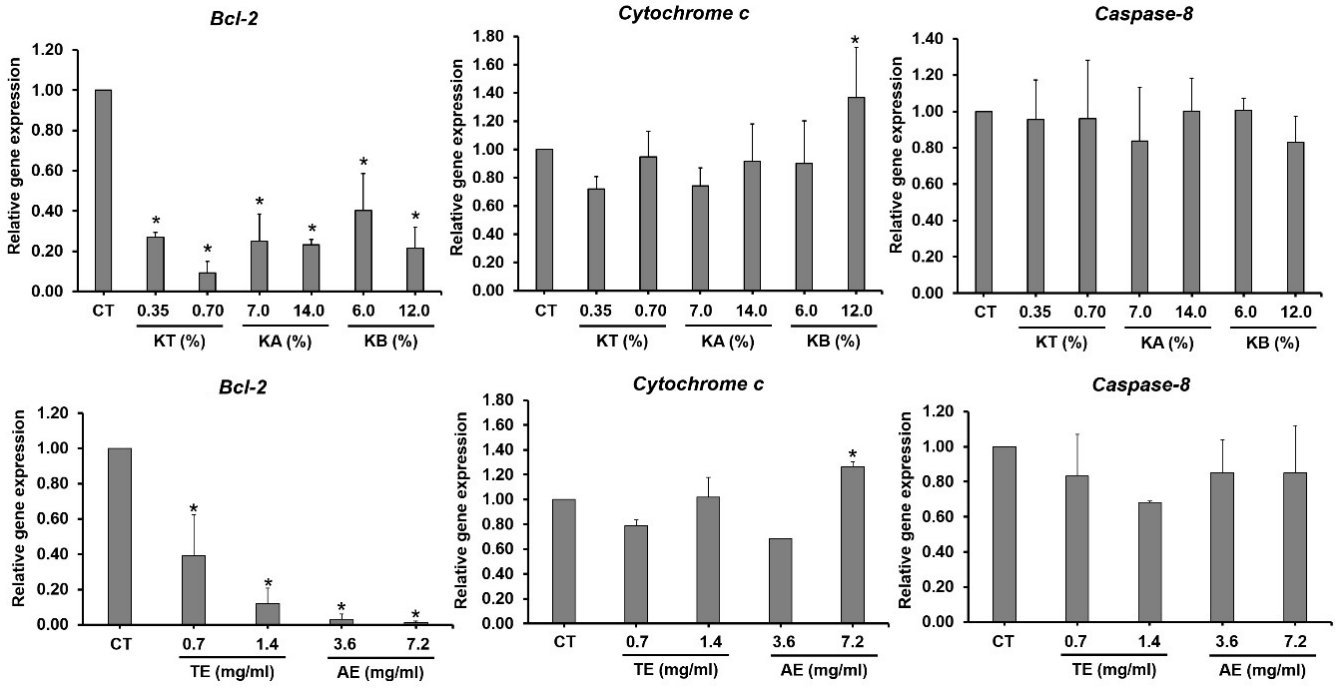

Figure 3. Relative gene expression related apoptosis pathway mechanism through *Bcl-2*, *cytochrome c* and *caspase-8* genes after treatment of Caco-2 cells with each concentration of kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), and kombucha prepared from black tea (KB), *T. catappa* extract (TE), and *A. marmelos* extract (AE) for 1 h. Gene expression ratios of Caco-2 cells were normalized to the expression of the regulator gene and compared to untreated cells (value at *P <0.05). Values are mean ± standard deviation; n = 3 samples.

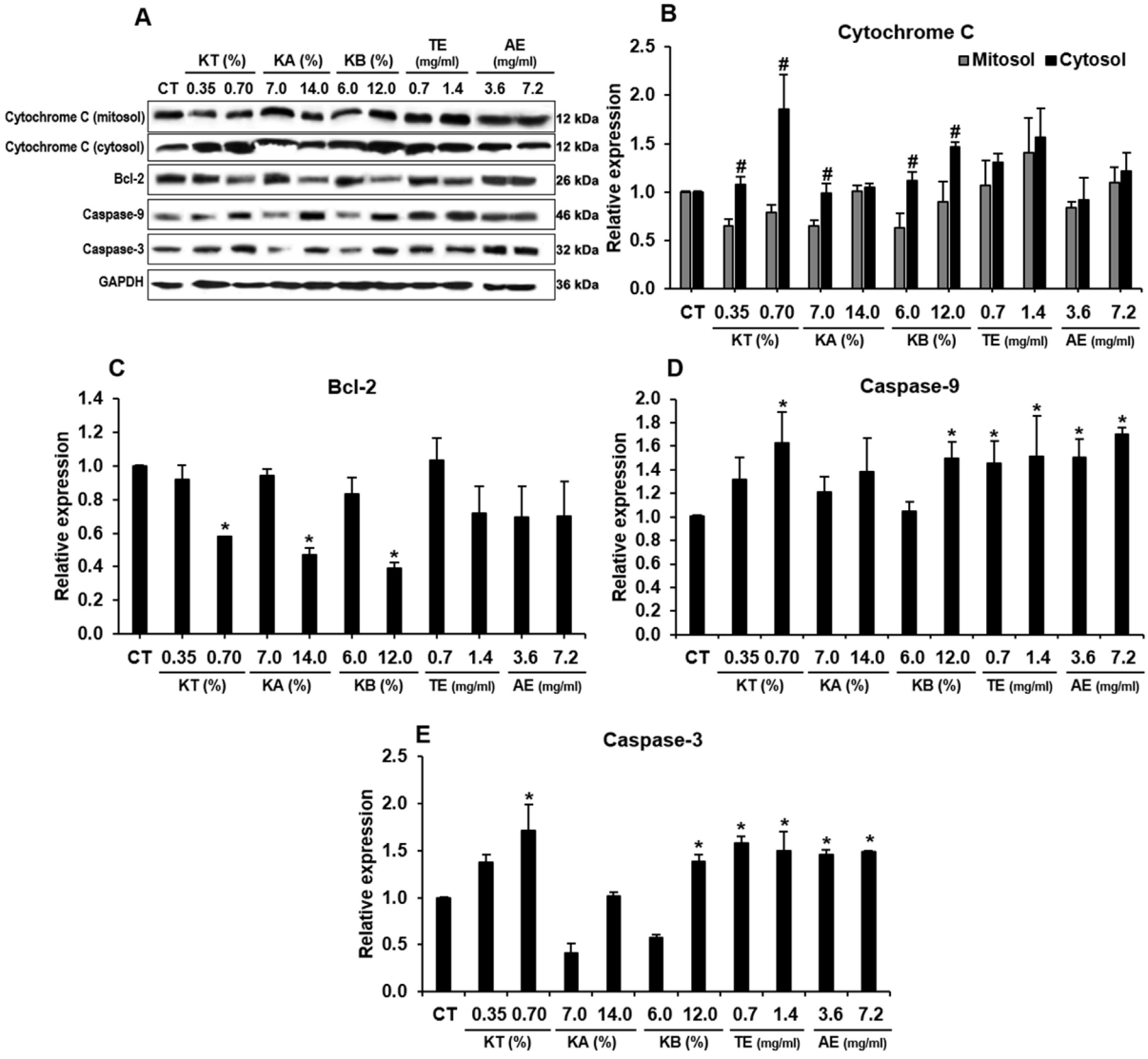

Figure 4. The apoptotic protein expression of Caco-2 cells after treatment with each concentration of kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), and kombucha prepared from black tea (KB), *T. catappa* extract (TE), and *A. marmelos* extract (AE) when compared with control cells (CT) for 1 h (*P <0.05). Proteins were detected with specific anti-apoptotic protein antibodies (cytochrome c, Bcl-2, caspase-9, and caspase-3). GAPDH protein was used as the internal protein loading control. (A) The apoptotic protein expression images are shown and (B-E) the relative expressions of the apoptotic protein are shown in bar graph. #P <0.05 comparing to cytochrome c in mitosol cells.

previous work revealed that kombucha prepared from black tea demonstrated various beneficial organic acids higher than kombucha prepared from green tea and oolong tea after microbial fermentation (Kaewkod et al., 2019). Medicinal plants *T. catappa* (leaf) and *A. marmelos* (fruit) were selected to enhance the beneficial properties of the tea in terms of antioxidant activity and the phytochemical compounds, as well as to enhance the anticancer properties. In the combination of kombucha with *T. catappa* (KT) and kombucha with *A. marmelos* (KA), antioxidant activity, phenolic compounds, flavonoids contents, and organic acids were all significantly increased when compared to the kombucha prepared without the plant extracts (KB). The potential beneficial aspects of traditional kombucha tea were found to have been vastly improved after being combined with the medicinal plant extracts. Likewise, the study of Shahbazi et al. (2018) developed kombucha tea containing cinnamon. This blend of kombucha tea displayed high antioxidant and antimicrobial activities, revealed greater organic acid contents, and exhibited improved sensorial scores. Moreover, after traditional kombucha tea was mixed with wheatgrass juice, it was found to enhance the amounts of phenolic compounds and antioxidant activities (Sun et al., 2015). The kombucha prepared from mint tea showed the highest phenolic content and general acceptability from sensory evaluation (Kayisoglu & Coskun, 2021).

In this study, the highest amount of beneficial substances were revealed in the KT extract followed by the KA extract. Furthermore, the amounts of organic acids (glucuronic acid, gluconic acid, D-saccharic acid 1,4-lactone (DSL), acetic acid, ascorbic acid, and succinic acid) were increased after the traditional kombucha tea was combined with plant extracts. KT was found to contain significantly

greater amounts of glucuronic acid, gluconic acid, DSL, ascorbic acid, and succinic acid than KA. Moreover, the highest amount of acetic acid was recorded in KA. These results demonstrated that *T. catappa* and *A. marmelos* extracts were also found to enhance the benefits of the organic acids existing in traditional kombucha tea. The extract of *T. catappa* contained six organic acids, namely glucuronic acid, gluconic acid, DSL, acetic acid, ascorbic acid, and succinic acid. Four organic acids (glucuronic acid, gluconic acid, acetic acid, and succinic acid) were found to be present in the *A. marmelos* extract in this study. This is a novel finding with regard to the reporting of some of the beneficial organic acids present in these medicinal plants. The plant species of *T. catappa* was found to be closely related to *T. ferdinandiana*, which contained ascorbic acid, ellagic acid, and oxalic acid (Williams et al., 2016). In the study conducted by Yadav et al. (2011), it was reported that bale fruit (*A. marmelos*) can be considered a natural medicinal fruit that contains a number of beneficial components such as polyphenol (tannin), vitamins (riboflavin and ascorbic), organic acids (oxalic, tartaric, and malic), and sugars (fructose, glucose, and sucrose). The increase in the organic acids present in the kombucha combined with medicinal plant extracts, particularly glucuronic acid, gluconic acid, DSL, and ascorbic acid, displayed a number of beneficial effects in both in vitro and in vivo studies. The glucuronic acid present in kombucha tea is associated with an important process involved in liver detoxification and the excretion of an exogenous chemical called glucuronidation (Ilmara et al., 2013). Notably, DSL, as an antioxidant molecule, could decrease oxidative damage, lipoprotein (LDL)-cholesterol, and serum estrogen levels in cells (Walaszek et al., 1996; Saluk-Juszczak et al., 2008). Ascorbic acid, also known as vitamin C, is a potent antioxidant that enhances the immune defense system by supporting several cellular functions of the innate and the adaptive immune system (Carr & Maggini, 2017).

Interestingly, kombucha combined with *T. catappa* (KT) and kombucha combined with *A. marmelos* (KA) displayed inhibitory activity against Caco-2 cancer cells that were greater than traditional kombucha tea (KB). This phenomenon correlated with the number of active substances present in the kombucha tea blends in the way of the phenolic compounds, flavonoids, antioxidative agents and organic acids. These exist in KT in higher amounts than in KA. Previous reports have demonstrated that total phenolics and flavonoid compounds in plant extracts have anticancer activity. Furthermore, these compounds could effectively moderate the anticancer effects on the human breast cancer (T47-D) cell line (Akillilar et al., 2018). Moreover, kombucha tea could inhibit the human tumor cell lines such as lung adenocarcinoma (A549) and liver hepatocellular carcinoma (HepG-2) (Deghrigue et al., 2013). The anticancer properties of kombucha tea have been reported to be attributed to the polyphenols and organic acids present in the tea. Polyphenols in kombucha tea were found to act as cancer-blocking agents that could reduce the process of cancer formation (Russo, 2007). Additionally, the organic acids in kombucha tea, such as DSL and ascorbic acid, were also revealed to exhibit anticancer properties (Hemilä & Herman, 1995; Wang et al., 2010). After Caco-2 cells were treated with KT and KA, apoptosis could be induced and DNA fragmentation appeared within the cancer cells. Similarly, the TUNEL assay also confirmed the effectiveness of KT and KA in the form of damage to the DNA of the Caco-2 cells. The pipeline of the DNA cleavage was expressed after the activation of the apoptotic caspase enzyme cascade and ultimately resulted in cell death. Furthermore, tea polyphenols investigated in previous research studies were found to be associated with the inhibition of gene mutation and cancer cell proliferation, the induction of cancer cell apoptosis, and the termination of metastasis (Conney et al., 2002; Ioannides & Yoxall, 2003; Park & Dong, 2003). Additionally, black tea could induce DNA strand breaks and oxidative damage to the DNA in carcinoma cells HT-29 and MCF-7 after being treated with black tea for 72 h (Koňariková et al., 2015). Moreover, organic acids, such as ascorbic acid, DSL, and succinic acid, are known to play an important role in apoptosis by induction of the DNA fragmentation of cancer cells through the activation of caspase enzymes (Shiau et al., 2006; Hong et al., 2007; Bhattacharya et al., 2013). Other compounds displaying anticancer activity that can be obtained from plant extracts are known to belong to phenolic and flavonoid groups. Quercetin has displayed powerful antioxidant activity due to its presence in flavonoids group (Vijayalakshmi et al., 2013). Moreover, quercetin could significantly inhibit human breast cancer cells (Scambia et al., 1994; Du et al., 2010). The polyphenolic compound of tannin was also revealed to suppress proliferation in various types of cancer cells in vitro and induce cancer cell death by way of apoptosis (Sakagami et al., 2000; Yang et al., 2000).

The important role of apoptosis mechanisms by KT and KA were confirmed by the activation of the caspase enzyme in the cancer cells. The apoptotic proteins, including cytochrome c, Bcl-2, caspase-9, and caspase-3, were detected after treatment with KT and KA. Apoptosis occurred through two main pathways identified as the extrinsic and intrinsic pathways. The extrinsic pathway is activated by the interaction of death receptors and the activation of caspase-8, while the intrinsic pathway stimulated the release of cytochrome c from the mitochondria and activation of the death signal (Zapata et al., 2001). In this study, the mRNA expression of *caspase-8* was analyzed and the results demonstrated that the *caspase-8* gene expression levels were not different after being treated with KT and KA. Thus, KT and KA were not associated with the extrinsic pathway of apoptosis. In contrast, mRNA and the protein expression levels associated with Bcl-2 production were found to have decreased through involvement with the intrinsic pathway after being treated with KT and KA in a dose-dependent manner. In terms of supporting these results, a low level of Bcl-2 expression protein corresponded to the reduction of the Bcl-2-Bax complex via an increase in the Bax molecules. The Bax molecule is produced and translocated into the mitochondrial membrane. Here, it induces the opening of the mitochondrial permeability transition pore to allow for the release of cytochrome c and to trigger the caspase cascade activation leading to apoptosis (Strasser et al., 2000). After Caco-2 cells were treated with KT and KA, the expression of cytochrome c increased in the cytosolic fraction and decreased in the mitochondrial fraction. Additionally, there was an increase in the activation of caspase-9 and caspase-3 expression. These phenomena suggest the ability of KT and KA to induce apoptosis through the intrinsic pathway. These findings support the claim of the beneficial effects of kombucha tea combined with *T. catappa* and *A. marmelos* to induce apoptosis pathways in colorectal cancer through the activation of the mitochondrial dependent apoptosis signaling pathways. The activation of the caspase enzyme may have

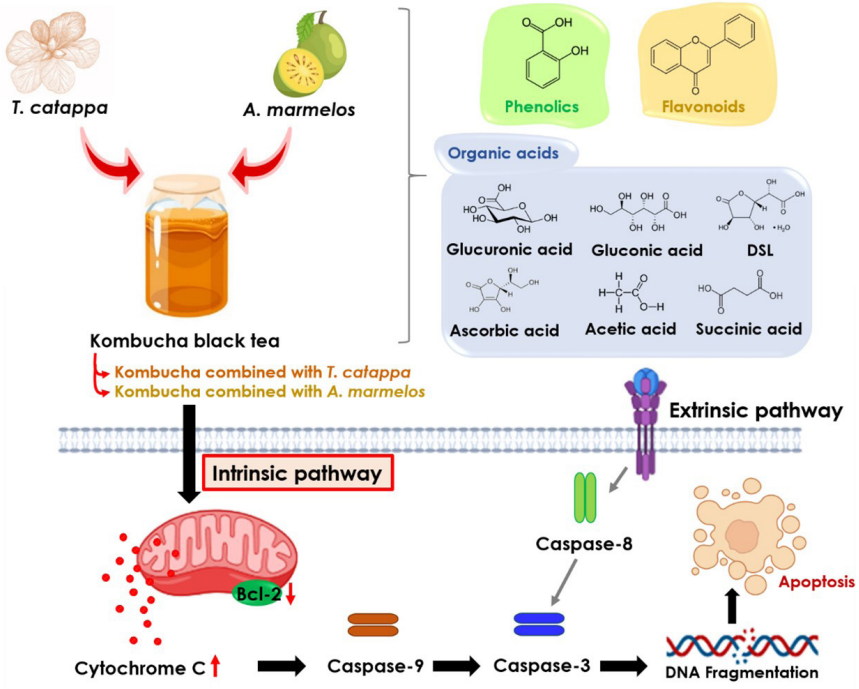

Figure 5. Proposed mechanism of kombucha combined with plant extracts to induce apoptosis pathway on colorectal cancer cell.

resulted from the chemical constituents of KT and KA. Kombucha tea was also found to inhibit the oxidative stress induced apoptosis pathway through the mitochondria-dependent pathways by cytochrome c release, activation of caspases-3 and 9, and Apaf-1 in murine hepatocytes that could prove to be beneficial against liver diseases (Bhattacharya et al., 2011). Furthermore, black tea, especially the compounds of theaflavins (TF) and thearubigins (TR), could induce apoptosis in human malignant melanoma A375 cells and Ehrlich's ascites carcinoma cells (EAC) through mediation of the mitochondria. The mechanism of cell death was determined to be initiated from the Bax translocation to the mitochondria and could induce the depolarization of the mitochondrial membrane potential and cytochrome c release in cytosol, followed by the activation of caspase-9, caspase-3, and poly (ADP-ribose) polymerase cleavage (Bhattacharyya et al., 2005; Halder et al., 2008).

The other effects contributing to the induction of the apoptosis pathways of cancer cells may have involved the organic acids present in kombucha tea. The study by Marques et al. (2013) reported on acetic acid as a prevention/therapeutic agent in colorectal carcinoma (CRC) treatment through lysosomal membrane permeabilization (LMP) induction and the subsequent release of cathepsin D (CatD) as an inhibitor for CRC treatment. Furthermore, the apoptosis promoted by ascorbic acid was associated with Bax and caspases activation, Bcl-xL sequestration, and cytochrome c release (Sant et al., 2018). This study also confirmed the effects of *T. catappa* and *A. marmelos*, which were mixed with kombucha tea. The apoptosis mechanisms of colorectal cancer Caco-2 cells observed in these plant extracts revealed similar functions through the intrinsic pathway. However, limited research has been performed on the mechanism of *T. catappa* and *A. marmelos* extracts in terms of their potential anticancer activity. It has been reported that ethanolic leaf extracts of *T. catappa* could significantly inhibit the migration of cells and the invasion capacities of squamous cell carcinoma 4 (SCC4) cells with regard to reducing the protein levels of MMP-2, MMP-9, and u-PA (Yang et al., 2010). Moreover, the phytochemical compound of 1-hydroxy-5,7-dimethoxy-2-naphthalene-carboxaldehyde obtained from *A. marmelos* exhibited an increase in activated caspase-3 levels and induced the G(1) cell cycle arrest of HCT-116 colon cancer. Additionally, these compounds could induce caspase-8, Bid activation, and cytochrome c release suggesting the role of the cross mechanisms between the death receptor and the mitochondrial pathways (Subramaniam et al., 2008). Moreover, apoptosis induction might be a result of phenolic and flavonoid compounds that were also found to be present in kombucha tea combined with plant extracts. The phenolic compound of tannin obtained from *Smilax china* L. rhizome increased the cleaved-caspase-3 protein expression in human lung adenocarcinoma A549 cells, followed by the activation of caspase-8 and caspase-9. Furthermore, flavonoids and tannins induced the expression of Bax and cytochrome c release, while the expression of Bcl-2 decreased (Fu et al., 2017).

5 Conclusion

This study revealed the enhanced properties of traditional kombucha tea through combinations with the medicinal plant extracts of *T. catappa* and *A. marmelos*. These medicinal plants could be employed to increase the activity of certain beneficial substances such as organic acids, antioxidant activities, phenolics, and various flavonoid compounds. Furthermore, KT and KA were found to exhibit cytotoxicity against Caco-2 cells and promoted the intrinsic apoptosis mechanisms (Figure 5). Therefore, traditional

kombucha combined with certain medicinal plants, namely *T. catappa* and *A. marmelos*, supported the beneficial aspects of these substances along with their anti-CRC activities. These new findings have demonstrated the significant potential health benefits of kombucha tea and certain enhanced properties of traditional kombucha tea through combinations with medicinal plant extracts. Hence, KT and KA could be employed as natural supplementary beverage products in the treatment and prevention of colorectal cancer.

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References

Akillilar, P. T., Tuglu, Y. B., & Moghaddam, N. S. (2018). Anticancer, antioxidant properties and phenolic, flavonoid composition of Heracleum platytaenium plant methanolic extracts. *Marmara Pharmaceutical Journal*, 22(3), 396-404.

Anand, A. V., Divya, N., & Kotti, P. P. (2015). An updated review of *Terminalia catappa. Pharmacognosy Reviews*, 9(18), 93-98. http://dx.doi.org/10.4103/0973-7847.162103. PMid:26392705.

Baliga, M. S., Bhat, H. P., Joseph, N., & Fazal, F. (2011). Phytochemistry and medicinal uses of the bael fruit (*Aegle marmelos* Correa): a concise review. *Food Research International*, 44(7), 1768-1775. http://dx.doi.org/10.1016/j.foodres.2011.02.008.

Bhattacharya, S., Gachhui, R., & Sil, P. C. (2011). Hepatoprotective properties of kombucha tea against TBHP-induced oxidative stress via suppression of mitochondria- dependent apoptosis. *Pathophysiology*, 18(3), 221-234. http://dx.doi.org/10.1016/j.pathophys.2011.02.001. PMid:21388793.

Bhattacharya, S., Gachhui, R., & Sil, P. C. (2013). The prophylactic role of D-saccharic acid-1,4-lactone against hyperglycemia-induced hepatic apoptosis via inhibition of both extrinsic and intrinsic pathways in diabetic rats. *Journal of Functional Foods*, 4(2), 283-296. http://dx.doi.org/10.1039/C2FO30145H. PMid:23138840.

Bhattacharyya, A., Lahiry, L., Mandal, D., Sa, G., & Das, T. (2005). Black tea induces tumor cell apoptosis by Bax translocation, loss in mitochondrial transmembrane potential, cytochrome c release and caspase activation. *International Journal of Cancer*, 117(2), 308-315. http://dx.doi.org/10.1002/ijc.21075. PMid:15880367.

Carr, A. C., & Maggini, S. (2017). Vitamin C and immune function. *Nutrients*, 9(11), 1-25. http://dx.doi.org/10.3390/nu9111211. PMid:29099763.

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. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.12.015. PMid:26796581.

Chan, E. W. C., Tie, P. P., Soh, E. Y., & Law, Y. P. (2011). Antioxidant and antibacterial properties of green, black and herbal teas of *Camellia sinensis. Pharmacognosy Research*, 3(4), 266-272. http://dx.doi.org/10.4103/0974-8490.89748. PMid:22224051.

Chitmanat, C., Tongdonmuan, K., Khanom, P., Pachontis, P., & Nunsong, W. (2005). Antiparasitic, antibacterial, and antifungal activities derived from a *Terminalia catappa* solution against some Tilapia (*Oreochromis niloticus*) pathogens. *Acta Horticulturae*, (678), 179-182. http://dx.doi.org/10.17660/ActaHortic.2005.678.25.

Conney, A. H., Lu, Y. P., Lou, Y. R., & Huang, M. T. (2002). Inhibitory effects of tea and caffeine on UV-induced carcinogenesis: relationship to enhanced apoptosis and decreased tissue fat. *European Journal of Cancer Prevention*, 11(Suppl. 2), S28-S36. PMid:12570332.

Deghrigue, M., Chriaa, J., Battikh, H., Abid, K., & Bakhrouf, A. (2013). Antiproliferative and antimicrobial activities of Kombucha tea. *African Journal of Microbiological Research*, 7, 3466-3470.

Du, G., Lin, H., Wang, M., Zhang, S., Wu, X., Lu, L., Ji, L., & Yu, L. (2010). Quercetin greatly improved therapeutic index of doxorubicin against 4T1 breast cancer by its opposing effects on HIF-1α in tumor and normal cells. *Cancer Chemotherapy and Pharmacology*, 65(2), 277-287. http://dx.doi.org/10.1007/s00280-009-1032-7. PMid:19466611.

Dufresne, C., & Farnworth, E. (2000). Tea, Kombucha, and health. *Food Research International*, 33(6), 409-421. http://dx.doi.org/10.1016/S0963-9969(00)00067-3.

Fu, S., Yang, Y., Liu, D., Luo, Y., Ye, X., Liu, Y., Chen, X., Wang, S., Wu, H., Wang, Y., Hu, Q., & You, P. (2017). Flavonoids and tannins from *Smilax china* L. rhizome induce apoptosis via mitochondrial pathway and mdm2-p53 signaling in human lung adenocarcinoma cells. *The American Journal of Chinese Medicine*, 45(2), 369-384. http://dx.doi.org/10.1142/S0192415X17500239. PMid:28231749.

Ge, X., Luo, X., Chen, Y., Li, M., Jiang, S., & Wang, X. (2011). Rhein induces apoptosis of HCT-116 human colon cancer cells via activation of the intrinsic apoptotic pathway. *African Journal of Biotechnology*, 10(61), 13244-13251.

Goldstein, D. (2015). *The Oxford companion to sugar and sweets* (1st ed.). Oxford: Oxford University Press.

Halder, B., Bhattacharya, U., Mukhopadhyay, S., & Giri, A. K. (2008). Molecular mechanism of black tea polyphenols induced apoptosis in human skin cancercells: involvement of Bax translocation and mitochondria mediated death cascade. *Carcinogenesis*, 29(1), 129-138. http://dx.doi.org/10.1093/carcin/bgm233. PMid:17984116.

Hemilä, H., & Herman, Z. (1995). Vitamin C and the common cold: a retrospective analysis of chalmers review. *Journal of the American College of Nutrition*, 14(2), 116-123. http://dx.doi.org/10.1080/07315724.1995.10718483. PMid:7790685.

Hong, S. W., Jin, D. H., Hahm, E. S., Yim, S. H., Lim, J. S., Kim, K. I., Yang, Y., Lee, S. S., Kang, J. S., Lee, W. J., Lee, W. K., & Lee, M. S. (2007). Ascorbate (vitamin C) induces cell death through theapoptosis-inducing factor in human breast cancer cells. *Oncology Reports*, 18(4), 811-815. PMid:17786340.

Ilmara, V., Raimonds, L., Arturs, P., & Pavels, S. (2013). Glucuronic acid from fermented beverages: biomedical function in humans and its role in health protection. *International Journal of Research and Reviews in Applied Sciences*, 14(2), 218-230.

Ioannides, C., & Yoxall, V. (2003). Antimutagenic activity of tea: role of polyphenols. *Current Opinion in Clinical Nutrition and Metabolic Care*, 6(6), 649-656. http://dx.doi.org/10.1097/00075197-200311000-00008. PMid:14557796.

Jafari, R., Naghavi, N. S., Khosravi-Darani, K., Doudi, M., & Shahanipour, K. (2021). Isolation, molecular and phylogenetic identification of microorganisms from Kombucha solution and evaluation of their viability using flow cytometery. *Food Science and Technology*. In press. http://dx.doi.org/10.1590/fst.63220.

Jayabalan, R., Malbasa, R. V., Loncar, 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. http://dx.doi.org/10.1111/1541-4337.12073. PMid:33412713.

Kaewkod, T., Bovonsombut, S., & Tragoolpua, T. (2019). Efficacy of kombucha obtained from green, oolong, and black teas on inhibition of pathogenic bacteria, antioxidation, and toxicity on colorectal cancer cell line. *Microorganisms*, 7(12), 1-18. http://dx.doi.org/10.3390/microorganisms7120700. PMid:31847423.

Kayisoglu, S., & Coskun, F. (2021). Determination of physical and chemical properties of kombucha teas prepared with different herbal teas. *Food Science and Technology*, 41(1, Suppl. 1), 393-397. http://dx.doi.org/10.1590/fst.12720.

Kim, S. K., Park, K. Y., Yoon, W. C., Park, S. H., Park, K. K., Yoo, D. H., & Choe, J. Y. (2011). Melittin enhances apoptosis through suppression of IL-6/sIL-6R complex-induced NF-κB and STAT3 activation and Bcl-2 expression for human fibroblast-like synoviocytes in rheumatoid arthritis. *Joint, Bone, Spine*, 78(5), 471-477. http://dx.doi.org/10.1016/j.jbspin.2011.01.004. PMid:21354845.

Koňariková, K., Ježovičová, M., Keresteš, J., Gbelcová, H., Ďuračková, Z., & Žitňanová, I. (2015). Anticancer effect of black tea extract in human cancer cell lines. *SpringerPlus*, 4(1), 127. http://dx.doi.org/10.1186/s40064-015-0871-4. PMid:25825685.

Lee, K. R., Jo, K., Ra, K. S., Suh, H. J., & Hong, K. (2021). Kombucha fermentation using commercial kombucha pellicle and culture broth as starter. *Food Science and Technology*. http://dx.doi.org/10.1590/fst.70020.

Marques, C., Oliveira, C. S. F., Alves, S., Chaves, S. R., Coutinho, O. P., Côrte-Real, M., & Preto, A. (2013). Acetate-induced apoptosis in colorectal carcinoma cells involves lysosomal membrane permeabilization and cathepsin D release. *Cell Death & Disease*, 4(2), e507. http://dx.doi.org/10.1038/cddis.2013.29. PMid:23429293.

Miliauskas, G., Venskutonis, P. R., & van Beek, T. A. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry*, 85(2), 231-237. http://dx.doi.org/10.1016/j.foodchem.2003.05.007.

Park, A. M., & Dong, Z. (2003). Signal transduction pathways: targets for green and black tea polyphenols. *Journal of Molecular Biology and Biochemistry*, 6(1), 66-77. PMid:12542977.

Russo, G. L. (2007). Ins and outs of dietary phytochemicals in cancer chemoprevention. *Biochemical Pharmacology*, 74(4), 533-544. http://dx.doi.org/10.1016/j.bcp.2007.02.014. PMid:17382300.

Sakagami, H., Jiang, Y., Kusama, K., Atsumi, T., Ueha, T., Toguchi, M., Iwakura, I., Satoh, K., Ito, H., Hatano, T., & Yoshida, T. (2000). Cytotoxic activity of hydrolyzable tannins against human oral tumor cell lines: a possible mechanism. *Phytomedicine*, 7(1), 39-47. http://dx.doi.org/10.1016/S0944-7113(00)80020-3. PMid:10782489.

Saluk-Juszczak, J., Olas, B., Nowak, P., Staroń, A., & Wachowicz, B. (2008). Protective effects of D-glucaro 1,4-lactone against oxidative modifications in blood platelets. *Nutrition, Metabolism, and Cardiovascular Diseases*, 18(6), 422-428. http://dx.doi.org/10.1016/j.numecd.2007.02.016. PMid:17933501.

Sandor, E. K. (2012). *The art of fermentation: an in-depth exploration of essential concepts and processes from around the world* (1st ed.). Vermont: Chelsea Green Publishing.

Sant, D. W., Mustafi, S., Gustafson, C. B., Chen, J., Slingerland, J. M., & Wang, G. (2018). Vitamin C promotes apoptosis in breast cancer cells by increasing TRAIL expression. *Scientific Reports*, 8(1), 5306. PMid:29593282.

Scambia, G., Ranelletti, F. O., Panici, P. B., De Vincenzo, R., Bonanno, G., Ferrandina, G., Piantelli, M., Bussa, S., Rumi, C., Cianfriglia, M., & Mancuso, S. (1994). Quercetin potentiates the effect of adriamycin in a multidrug-resistant MCF-7 human breast-cancer cell line: P-glycoprotein as a possible target. *Cancer Chemotherapy and Pharmacology*, 34(6), 459-464. http://dx.doi.org/10.1007/BF00685655. PMid:7923555.

Shahbazi, H., Hashemi Gahruie, H., Golmakani, M.-T., Eskandari, M. H., & Movahedi, M. (2018). Effect of medicinal plant type and concentration on physicochemical, antioxidant, antimicrobial, and sensorial properties of kombucha. *Food Science & Nutrition*, 6(8), 2568-2577. http://dx.doi.org/10.1002/fsn3.873. PMid:30510759.

Shiau, C. W., Huang, J. W., Wang, D. S., Weng, J. R., Yang, C. C., Lin, C. H., Li, C., & Chen, C. S. (2006). Tocopheryl succinate induces apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 function. *The Journal of Biological Chemistry*, 281(17), 11819-11825. http://dx.doi.org/10.1074/jbc.M511015200. PMid:16520381.

Singhatong, S., Leelarungrayub, D., & Chaiyasut, C. (2010). Antioxidant and toxicity activities of *Artocarpus lakoocha* Roxb. Heartwood extract. *Journal of Medicinal Plants Research*, 4(10), 947-953.

Strasser, A., O'Connor, L., & Dixit, V. W. (2000). Apoptosis signaling. *Annual Review of Biochemistry*, 69(1), 217-245. http://dx.doi.org/10.1146/annurev.biochem.69.1.217. PMid:10966458.

Subhadrabandhu, S. (2001). *Under-utilized tropical fruits of Thailand* (1st ed.). Bangkok: Food and Agriculture Organization of the United Nations Regional Office for Asia and the Pacific.

Subramaniam, D., Giridharan, P., Murmu, N., Shankaranarayanan, N. P., May, R., Houchen, C. W., Ramanujam, R. P., Balakrishnan, A., Vishwakarma, R. A., & Anant, S. (2008). Activation of apoptosis by 1-hydroxy-5,7-dimethoxy-2-naphthalene-carboxaldehyde, a novel compound from Aegle marmelos. *Cancer Research*, 68(20), 8573-8581. http://dx.doi.org/10.1158/0008-5472.CAN-08-2372. PMid:18922933.

Sun, K. W., Ma, Y. Y., Guan, T. P., Xia, Y. J., Shao, C. M., Chen, L. G., Ren, Y. J., Yao, H. B., Yang, Q., & He, X. J. (2012). Oridonin induces apoptosis in gastric cancer through Apaf-1, cytochrome c and caspase-3 signaling pathway. *World Journal of Gastroenterology*, 18(48), 7166-7174. http://dx.doi.org/10.3748/wjg.v18.i48.7166. PMid:23326121.

Sun, T. Y., Li, J. S., & Chen, C. (2015). Effects of blending wheatgrass juice on enhancing phenolic compounds and antioxidant activities of traditional kombucha beverage. *Journal of Food and Drug Analysis*, 23(4), 709-718. PMid:28911487.

Teoh, A. L., Heard, G., & Cox, J. (2004). Yeast ecology of kombucha fermentation. *International Journal of Food Microbiology*, 95(2), 119-126. http://dx.doi.org/10.1016/j.ijfoodmicro.2003.12.020. PMid:15282124.

Umthong, S., Phuwapraisirisan, P., Puthong, S., & Chanchao, C. (2011). In vitro antiproliferative activity of partially purified Trigona laeviceps propolis from Thailand on human cancer cell lines. *BMC Complementary and Alternative Medicine*, 11(1), 37. http://dx.doi.org/10.1186/1472-6882-11-37. PMid:21548933.

Venugopal, R., & Liu, R. H. (2012). Phytochemicals in diets for breast cancer prevention: The importance of resveratrol and ursolic acid. *Food Science and Human Wellness*, 1(1), 1-13. http://dx.doi.org/10.1016/j.fshw.2012.12.001.

Vijayalakshmi, A., Kumar, P. R., Priyadarsini, S. S., & Meenaxshi, C. (2013). In Vitro antioxidant and anticancer activity of flavonoid fraction from the aerial parts of Cissus quadrangularis Linn. against human breast carcinoma cell lines. *Journal of Chemistry*, 2013, 1-9. http://dx.doi.org/10.1155/2013/150675.

Walaszek, Z., Szemraj, J., Hanausek, M., Adams, A. K., & Sherman, U. (1996). D-glucaric acid content of various fruits and vegetables and cholesterol-lowering effects of dietary D-glucarate in the rat. *Nutrition Research (New York, N.Y.)*, 16(4), 673-681. http://dx.doi.org/10.1016/0271-5317(96)00045-0.

Wang, K., Gan, X., Tang, X., Wang, S., & Tan, H. (2010). Determination of D-saccharic acid- 1,4–lactone from brewed kombucha broth by high performance capillary electrophoresis. *Journal of Chromatography A*, 878(3-4), 371-374. PMid:20015711.

Williams, D., Edwards, D., Pun, S., Chaliha, M., Burren, B., Tinggi, U., & Sultanbawa, Y. (2016). Organic acids in Kakadu plum (Terminalia ferdinandiana): The good (ellagic), the bad (oxalic) and the uncertain (ascorbic). *Food Research International*, 89(1), 237-244. http://dx.doi.org/10.1016/j.foodres.2016.08.004. PMid:28460910.

Williamson, G., Barron, D., Shimoi, K., & Terao, J. (2005). In vitro biological properties of flavonoid conjugates found in vivo. *Free Radical Research*, 39(5), 457-469. http://dx.doi.org/10.1080/10715760500053610. PMid:16036321.

Yadav, N., Tyagi, G., Jangir, D. K., & Mehrotra, R. (2011). Rapid determination of polyphenol, vitamins, organic acids and sugars in *Aegle marmelos* using reverse phase-high performance liquid chromatography. *Journal of Pharmacy Research*, 4(3), 717-719.

Yang, L. L., Lee, C. Y., & Yen, K. Y. (2000). Induction of apoptosis by hydrolyzable tannins from *Eugenia jambos* L. on human leukemia cells. *Cancer Letters*, 157(1), 65-75. http://dx.doi.org/10.1016/S0304-3835(00)00477-8. PMid:10893444.

Yang, S. F., Chen, M. K., Hsieh, Y. S., Yang, J. S., Zavras, A. I., Hsieh, Y. H., Su, S. C., Kao, T. Y., Chen, P. N., & Chu, S. C. (2010). Anti-metastatic effects of *Terminalia catappa* L. on oral cancer via a down-regulation of metastasis-associated proteases. *Food and Chemical Toxicology*, 48(4), 1052-1058. http://dx.doi.org/10.1016/j.fct.2010.01.019. PMid:20102732.

Yildiz, E., Guldas, M., & Gurbuz, O. (2021). Determination of in-vitro phenolics, antioxidant capacity and bio-accessibility of Kombucha tea produced from black carrot varieties grown in Turkey. *Food Science and Technology*, 41(1), 180-187. http://dx.doi.org/10.1590/fst.00320.

Zapata, J. M., Pawlowski, K., Haas, E., Ware, C. F., Godzik, A., & Reed, J. C. (2001). A diverse family of proteins containing tumor necrosis factor receptor-associated factor domains. *The Journal of Biological Chemistry*, 276(26), 24242-24252. http://dx.doi.org/10.1074/jbc.M100354200. PMid:11279055.

Zar, P. P., Yano, S., Sakao, K., Hashimoto, F., Nakano, T., Fujii, M., & Hou, D. X. (2014). In vitro anticancer activity of loquat tea by inducing apoptosis in human leukemia cells. *Bioscience, Biotechnology, and Biochemistry*, 78(10), 1731-1737. http://dx.doi.org/10.1080/09168451.2014.936352. PMid:25118018.

Supplementary Material

Supplementary material accompanies this paper.

Figure 1S. DNA fragmentation of Caco-2 cells after treatment with each concentration of kombucha combined with *T. catappa* (KT), kombucha combined with *A. marmelos* (KA), and kombucha prepared from black tea (KB), *T. catappa* extract (TE), and *A. marmelos* extract (AE) for 24 h when compared with control cells by agarose gel electrophoresis.

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