Trop J Nat Prod Res, March 2023; 7(3):2536-2539 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Antioxidant Activity, Physicochemical Characterisation and Antibacterial Properties of Caspian Sea Yoghurt Enriched with Ginger and Sappanwood Extracts

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ARTICLE INFO

Article history:
Received 24 October 2022
Revised 15 March 2023
Accepted 17 March 2023
Published online 01 April 2023

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ABSTRACT

Caspian Sea yoghurt has lower acidity and is thicker than common yoghurt. Caspian Sea yoghurt contains probiotic bacteria that improve the balance of intestinal microflora. This research aimed to determine the antioxidant activity of Caspian sea yoghurt enriched with two different herbs, ginger and sappanwood, at concentrations of 5, 10 and 15% w/v. The pH, total phenol, antioxidant, total LAB, and antibacterial activity of the enriched yoghurt were measured to determine the best of the two products. The results showed that the enrichment of the yoghurt with sappanwood and ginger extracts increased lactic acid bacteria (LAB) growth compared with the control (yoghurt without plant extracts). Further analysis showed that the product that contains 15% sappanwood extract was judged to be the best formulation with a pH of 5.01 and a total LAB of 3.78 x 10^7 CFU/mL. It also exhibited antibacterial activity against *B. cereus* and *E. Coli* with zones of inhibition of 8.00 mm and 8.39 mm, respectively. The total phenol content was 5.48 mg GAE/g of the sample and 50.08% antioxidant activity. In conclusion, Caspian sea yoghurt enriched with herbs showed increased antioxidant and antibacterial activities and may have better potential in health maintenance.

Keywords: Caspian Sea Yogurt, Ginger, Sappan wood, Antioxidant, Antibacterial.

Introduction

Several species of plants in Indonesia are used as raw materials for traditional medicine or herbal treatments. Herbal medicine has been used for thousand years[1]and this knowledge has become a special tradition and culture in rural communities. Herbal medicine could be defined as plants or some parts of the plant used for their scent, flavour, colour, or other therapeutic properties.[2,3] The World Health Organization (WHO) estimated that up to 80% of the world's population (four billion people) use one or more forms of herbal medicine.[1,2] Herbal medicine could be used to prevent some diseases and cure chronic illnesses such as hypertension, diabetes, cancer, metabolic diseases and inflammatory bowel diseases.[4] It also has been suggested to have low side effects and toxicity due to the ability of the body to remove metabolites of herbal medicine easily.[5] There is a global increase in the use of herbal medicinal products to treat some diseases, spurred by the development of innovative herbal medicines.[6]

Plant species that have been used in herbal medicine include *Caesalpinia sappan* L. (sappanwood) and *Zingiber officinale* (ginger). Sappanwood contains gallic acid, tannins, resorcin, brazilin, brasilein, d-alpha-phellandrene, ocimene, alkaloids, flavonoids, saponins, phenyl propane, terpenoids, and essential oils.[7] Sappanwood is normally used as a natural pigment due to its ability to produce red pigments called anthocyanins, readily soluble in hot water.[8]

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Citation: Kusnadi J, Tirtania AR, Arumingtyas EL. Antioxidant Activity, Physicochemical Characterisation and Antibacterial Properties of Caspian Sea Yoghurt Enriched with ginger and Sappanwood Extracts. Trop J Nat Prod Res. 2023; 7(3):2536-2539 http://www.doi.org/10.26538/tjnpr/v7i3.11

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Sappanwood has been shown to be beneficial for health and exhibited antioxidant, antibacterial,[9] anti-inflammatory, antidiabetic, anti-anaemia, and neuroprotective activities.[7] Ginger has been used as a spice for at least 2000 years.[10] Its rhizomes have a pungent flavour, attributed to gingerol, a major phytoconstituents of the plant with known biological activity. Ginger contains several phytochemicals and phytonutrients: essential oils (2-3%), starch 40-60%, oleoresin, organic acids, malic acid, oxalic acid, gingerol, shogaols,[11] zingerone, flavonoids, polyphenols, alkaloids, and mucilage.[12] Ginger has been used to treat rheumatism, nervous diseases,[10] possess anti-diabetic, anti-inflammatory, antioxidant[13], antibacterial, and anticancer activities.[12]

Yoghurt is a dairy product that uses bacteria to increase the nutritional value of milk. Caspian Sea Yoghurt is widely used in Japan with unique characteristics, such as high viscosity and lower acidity, thus increasing consumer acceptance.[14] These attributes are possibly associated with the starter bacteria such as *Lactococcus lactis* subsp *cremoris* and *Acetobacter orientalis*, which can be used to create a distinctive taste in the yoghurt.[15] Recently, yoghurts with medicinal herbs incorporated in their formulation have been developed. Fortified yoghurt with herbal ingredients has increased nutritional value compared with regular yoghurt. This study evaluated the physicochemical and antioxidant properties of herbal Caspian Sea yoghurt formulation enriched with sappanwood and ginger extracts as a healthy drink.

Materials and Methods

Materials

The materials include pasteurised fresh milk (purchased from Merjosari traditional market, Malang, East Java, Indonesia), powdered bacterial cultures of Caspian sea yoghurt containing *Lactobacillus cremoris* and *Acetobacter orientalis* (Japora Corporation, Ltd, Hyogo, Japan) with patent number JP2004261176A, distilled water, NaOH, pH 4 and pH 7 buffers, de Man Rogosa Sharpe (MRS agar) media (Catalogue number 110660, Merck, Germany), peptone, 70% alcohol, 90% ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, Folin-ciocalteu reagent, and Na_2CO_3.

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Research design
This research was conducted using a Randomized Block Design with two factors of herbal extract, including types and addition concentrations. The herbal extracts were ginger and sappanwood at 5, 10, and 15% w/v concentrations. Caspian sea yoghurt without herbal addition was used as a control.

Caspian Sea Yogurt Starter Formulation
Sugar was added into pasteurised milk at 80-90°C for 15 minutes and cooled to 40°C. Furthermore, activated Caspian Sea Yogurt starter (5% v/v) was inoculated into 95 mL milk. The milk was incubated at room temperature (30 ± 1°C) for 12 h in the dark condition.[15]

Herbal extraction
The powdered ginger used for this study was obtained from Samudera Baraka, Surabaya Regency, with distribution permit no. 503/7219.A/436.7.17/2017 by the Indonesian National Agency of Drug and Food Control, while shaved sappanwood was from Ibtiqa, Bogor, East Java. The herbal materials were weighed and macerated in distilled water (ratio 1:10), heated at 65°C for 20 minutes and then cooled to 40°C. The extracts were kept in the refrigerator until used.
The herbal Caspian Sea yoghurt formulations were obtained by adding each extract into Caspian sea yoghurt at a concentration of 5, 10, and 15% w/v. The acidity or pH of the herbal Caspian sea yoghurts was analysed at 0, 2, 4, 6, 8, 12, and 24 hours of fermentation.

Measurement of total phenolic content (TPC) and antioxidant activity
Total Phenolic content (TPC) was measured using the Follin-Ciocalteu method described by Budiono *et al.* [16] with some modifications. Gallic acid (20 – 80 mg/mL) was used to prepare the calibration curve. 10 µL of the solution was added to the microplate reader containing 160 µL of distilled water. Ten microliters (10 µL) of 10% Folin-Ciocalteu reagent and 20 µL of 10% Na_2CO_3 solution were added to each well and incubated for 30 minutes at room temperature. The absorbance was measured using a spectrophotometer at 775 nm.

Lactic acid bacterial (LAB) analysis
The total LAB test was based on the method of Ummah et al.[17] with modifications. The sample (0.1 ml) was added to 0.9 ml of a 10^7 -10^9 CFU/mL dilution of fermented milk. Then 1 ml of each sample was grown in a deMan Rogosa Sharpe Agar (MRS agar) medium to determine the total amount of LAB. Petri dishes containing samples and media were incubated at 37ºC for 48 hours, and then the total LAB analysis results were calculated using the BAM (Bacteriological Analytical Manual) method.
LAB analysis: the amount of colonies x 1/10 x Log^{10}

Antibacterial activity
The antibacterial activity of the herbal yoghurt formulation was carried out using the agar well diffusion method.[18] Mueller Hinton'sAgar (MHA) media (20 mL) was transferred into the petri dishes and allowed to solidify. After that, the bacterial (*Escherichia coli* and *Bacillus cereus*) test suspension was flattened using a sterile cotton swab on the media. Then 5 holes of 6 mm diameter wells were made on the Petri dish. 50 µL of yoghurt sample was added to each well and then incubated for 24 hours. The antibacterial activity was indicated by clear zones formed around the well after 24 hours.

Statistical analysis
The data obtained were analysed using analysis of variance (ANOVA) with a confidence level of 5%, followed by the Least Significant Difference (LSD) test with a 5% confidence interval. The best treatment was selected using the Multiple Attribute Zeleny method.

Results and Discussion

Based on the data (Table 1), the TPC of powdered ginger was 0.19 mg/g GAE. A comparative study between ginger water extract (GWE) and ginger ethanolic extract (GEE) showed that the TPC levels of GWE and GEE were 52.8 and 137.5 mg/g GAE.[19] The different results might be caused by the difference in ginger cultivars, geographic distribution,[20] and extraction process leading to different levels of bioactive compounds. Meanwhile, the antioxidant capacity of powdered ginger was 51.51%. Other researchers reported that ginger rhizome's antioxidant was 51-58%.[21] Conversely, the TPC levels of sappanwood were 0.14 mg/g GAE or 443.20 to 885.12 mg/100 g extract, with antioxidant capacity reaching up to 55.13%.[22] The solvent polarity and extraction methods may have played a significant role in the TPC level in sappanwood.
Adding herbal ingredients to Caspian sea yoghurt increased the TPC levels and antioxidant capacity (Table 2). The higher phenol compounds could increase their function as antioxidants due to the ability to eliminate free and peroxide radicals, enhancing their effectiveness in inhibiting lipid oxidation.[16,23] The length of fermentation time also affected the antioxidant content. The longer time led to higher content and increased lactic acid content.[24]
The increase in extract concentration of the yoghurt produced a relatively stable pH (degree of acidity) (Table 3). The result suggested that adding herbal extract in various concentrations had no significant effect on the pH of Caspian sea yoghurt. Meanwhile, the results showed lower pH during the fermentation time.

Table 1: Total Phenol content and percentage Antioxidant activity of the test samples

Herbal	Raw Material	
	Total phenolic content (mg GAE/g sample)	**Antioxidant (%)**
Ginger	0.19	51.51
Sappanwood	0.14	55.13

Table 2: TPC and antioxidant capacity of herbal Caspian Sea Yogurt

Herbs types	Concentrations (% w/v)	Total phenol (mg GAE/g sample)	Antioxidant capacity during fermentation (%)	
			12 hours	24 hours
Control	0	5.63 ± 1.61	17.90 ± 6.16^{d}	35.02 ± 1.70^{d}
Powdered ginger	5	4.66 ± 0.71	23.11 ± 6.59^{cd}	38.20 ± 4.72^{cd}
	10	5.15 ± 1.72	23.25 ± 4.99^{cd}	39.21 ± 5.59^{cd}
	15	5.35 ± 1.65	33.89 ± 5.51^{bc}	46.24 ± 6.26^{bc}
Shaved sappanwood	5	2.89 ± 0.18	40.81 ± 7.53^{ab}	49.67 ± 3.32^{b}
	10	3.08 ± 0.17	47.34 ± 7.95^{ab}	67.51 ± 1.41^{a}
	15	5.48 ± 2.16	50.08 ± 5.35^{a}	70.54 ± 0.98^{a}

Data were presented as mean ± standard deviation. $^{a,b,c,d....}$different letters indicated a significant difference between groups

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Table 3: pH value of Caspian Sea Yogurt in different concentrations of herbs added during fermentation time

Herbs types	Concentrations (%)	Fermentation time						
		0	2	4	6	8	12	24
Control	0	7.25 ± 0.02	7.07 ± 0.01	6.85 ± 0.01	6.26 ± 0.02	5.34 ± 0.01	4.84 ± 0.02	4.65 ± 0.01
Powdered	5	7.14 ± 0.01	7.03 ± 0.01	6.73 ± 0.02	6.17 ± 0.01	4.75 ± 0.04	4.69 ± 0.02	4.61 ± 0.01
ginger	10	7.20 ± 0.02	6.51 ± 0.01	6.37 ± 0.02	6.23 ± 0.02	4.62 ± 0.01	4.61 ± 0.02	4.60 ± 0.01
	15	7.15 ± 0.01	6.99 ± 0.01	6.69 ± 0.01	5.94 ± 0.01	5.07 ± 0.01	4.88 ± 0.01	4.56 ± 0.01
Shaved	5	7.14 ± 0.01	7.01 ± 0.01	6.73 ± 0.01	6.05 ± 0.01	5.17 ± 0.01	4.80 ± 0.02	4.64 ± 0.01
sappanwood	10	7.12 ± 0.02	7.04 ± 0.01	6.75 ± 0.01	6.13 ± 0.04	5.37 ± 0.05	4.93 ± 0.02	4.63 ± 0.01
	15	7.18 ± 0.01	6.78 ± 0.02	6.41 ± 0.01	6.32 ± 0.01	5.61 ± 0.04	5.01 ± 0.01	4.66 ± 0.02

Data were presented as mean ± standard deviation

The change in pH during fermentation time may be due to the increase in the microbial activity in the yoghurt, which led to increased lactic acid levels, and this may have been responsible for the decrease in the pH.[24] The decrease in pH was due to the activity of lactic acid bacteria (LAB) to produce energy through the fermentation process by breaking down the substrate into simpler components. This lactose breakdown energy is combined with the lactic acid produced, decreasing the pH value. Lactic acid and acetaldehyde also cause a decrease in the pH of the fermentation medium to produce a distinctive aroma.

The LAB measurement showed significant differences between the original Caspian sea yoghurt (without herbal addition) and herbal Caspian sea yoghurt. The highest LAB content was observed in the formulation enriched with 15% w/v of powdered ginger (Table 4). Lactic acid is normally produced through the long fermentation process by LAB. LAB breaks down lactose into lactic acid during fermentation to produce a sour taste and increase the stability of fermented milk products such as cheese, yoghurt, and kefir.[25] The increase in the content of the added herbal extracts also led to an increase in the diameter of the inhibition zones (Table 5). This result is associated with increased antibacterial activity caused by an increased percentage of the herbal extracts. Moreover, the diameter of the clear zone was also affected by the difference in sensitivity of the test bacteria.

Gram-positive bacteria tend to be more sensitive to antibacterial agents due to their simple cell wall structure than Gram-negative bacteria, making it easier for antibacterial compounds to penetrate the rigid peptidoglycan layer and teichoic acid cell walls.[26] In contrast, the cell wall of Gram-negative bacteria consists of one or more layers of peptidoglycan and a membrane on the outside of the peptidoglycan, while the outer membrane has lipoproteins, phospholipids, and polysaccharides. This lipopolysaccharide component is essential because it indicates the level of toxicity in animals. The inseparability of the toxicity from the bacterial cells led to its designation as an endotoxin.[27] The mechanism of action of the bacteriocins found in yoghurt includes: inhibiting cell wall synthesis, protein synthesis, nucleic acid synthesis, and major metabolic pathways. Bacteriocins are known to produce large, clear, and round zones of inhibition making it possible for easy identification in potential antibacterial tests.[27]

The formulation of herbal Caspian sea yoghurt was determined using the Multiple Attribute Zeleny method with a focus on parameters such as pH, total LAB, antibacterial activity, total phenol, and antioxidant content. It was discovered that the best proportion was Caspian Sea Yogurt enriched with 15% w/v shaved sappanwood extract, which had a pH value of 5.01 and total LAB of 3.78 x 10^7, antibacterial zones of inhibition of 8.39 mm in *E. coli* and 8.00 mm in *B. cereus*, total phenol content of 5.48 g GAE/mL, and antioxidant percentage of 50.08%.

Table 4: Total LAB of Caspian Sea Yogurt in different concentrations of added herbs

Herbs types	Concentrations (%)	Number of colonies (10^7 CFU/mL)
Control	0	3.78 ± 0.58
Powdered ginger	5	4.00 ± 0.51
	10	3.34 ± 0.82
	15	4.67 ± 0.89*
Shaved sappanwood	5	4.43 ± 1.02*
	10	4.20 ± 0.29
	15	3.78 ± 1.27

Data were presented as mean ± standard deviation

Conclusion

The results showed that the enrichment of Caspian Sea Yogurt with medicinal herbs extract (ginger and sappanwood) increased its nutritional value compared to the control (without herbal extracts). The study also showed that sappanwood extract (15% w/v) resulted in the best formulation in all the parameters tested. Further research is necessary to observe the other herbal ingredients that could be used to fortify yoghurt products to improve their nutritional status.

Table 5: Antibacterial activity of Caspian Sea Yogurt in different extracts concentrations

Herbs types	Concentrations (%)	Diameter of zones of inhibition on each test bacteria (mm)	
		E. coli	*B. aureus*
Control	0	7.94 ± 0.89^{a}	7.44 ± 0.88^{a}
Powdered ginger	5	8.05 ± 1.26^{ab}	7.44 ± 0.89^{a}
	10	8.17 ± 1.21^{ab}	7.50 ± 0.68^{ab}
	15	8.50 ± 1.34^{a}	7.78 ± 1.02^{bc}
Shaved sappanwood	5	7.89 ± 1.31^{bc}	7.56 ± 0.64^{c}
	10	8.11 ± 1.23^{cd}	7.61 ± 0.83^{cd}
	15	8.39 ± 1.49^{d}	8.00 ± 1.09^{d}

Data were presented as mean ± standard deviation. $^{a,b,c,d,\cdots}$different letters indicated a significant difference between groups.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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