JFHB
Journal of Food Health and Bioenvironmental Science

Journal of Food Health and Bioenvironmental Science

Vol. 16 No. 1 January – April 2023

Journal of Food Health and Bioenvironmental Science

http://jfhb.dusit.ac.th/
http://research.dusit.ac.th/new/e-journal/

ISSN 2630-0311 (Online)

Journal of Food Health and Bioenvironmental Science

Vol. 16 No. 1

January – April 2023

ISSN 2630-0311 (Online)

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Journal of Food Health and Bioenvironmental Science

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Vol. 16 No. 1 January - April 2023 ISSN 2630-0311 (Online)

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Content

Journal of Food Health and Bioenvironmental Science
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Formulation and Quality Evaluation of Instant Sweet-Shrimp Paste (Kapi Wan) Powder

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Article info

Article history:
Received: 3 January 2023
Revised: 20 February 2023
Accepted: 20 March 2023

Keywords:
Sweet-shrimp paste, Instant powder, Drying, Product development

Abstract

The sweet-shrimp paste sauce (Kapi Wan) is a traditional Thai fruit dip prevalent in Southeast Asia. In this study, an instant sweet-shrimp paste powder was developed as a new product for convenience, portability and ease to use. The shrimp paste powder prepared by sun drying in a combination of hot air oven exhibited a low moisture content (8.53%) and a_w value (0.47) which preserved the character of natural color and flavor of the raw material. For an instant product preparation, the proportion of three ingredients, e.g., shrimp-paste powder (40-60%), palm sugar powder (10-30%) and cayenne pepper (10-30%), mixed with 30% ground dried shrimp (fixed ingredient) were studied. Choosing the most suitable formula from a total of 7 treatments based on the sensory attributes in terms of appearance, color, flavor, taste and overall liking by using the overlapping area of the contour plot of all factors. The best formula was treatment No. 1 (46.15% shrimp paste powder, 23.08% palm sugar powder, 7.69% cayenne pepper and 23.07% ground dried shrimp) which had the highest score in taste and overall liking. The product contained 12.01% of moisture content and the a_w value was 0.52. Total protein, fat, carbohydrate and ash contents were 33.52, 3.81, 31.49 and 19.01%, respectively. The second best treatment was Treatment No. 5 (41.03% shrimp paste powder, 17.95% palm sugar powder, 17.95% cayenne pepper and 23.07% ground dried shrimp), which showed similar physiochemical properties, except a significantly ($p \leq 0.05$) higher in fat (4.87%) and lower in ash (18.37%) content. Both treatments had the potential to develop an instant sweet-shrimp paste powder; as treatment No. 1 being a non-spicy and spicy formula as treatment No. 5. The sensory score ranged from like slightly to like moderately.

Introduction

The fermented shrimp paste is the most widely used condiment and seasoning ingredient in Southeast Asia. In Thailand it is commonly known as Kapi, while in other countries its prominent names include; Bagoong (Philippines), Prahok (Cambodia), Belacan (Malaysia), Ngapi (Myanmar), Mam ruoc (Vietnam) and Terasi

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(Indonesia). The making of fermented shrimp paste depends on the raw materials available in each nation (Hajeb & Jinap, 2012; Daroonpunt et al., 2016). Kapi is a thick, paste-like, purple-pink color, with a unique aroma, which is made by mixing the planktonic shrimp (Acetes sp. and Mesopodopsis sp.) with salt and then undergoes fermentation for several months (Daroonpunt et al., 2016; Kleechaya et al., 2021). In Thailand, shrimp paste is produced in many coastal areas as well as the estuary of the Gulf of Thailand in the provinces of Samut Songkhram, Samut Prakan, Samut Sakhon and Phetchaburi (Kleechaya et al., 2021).

The main characteristic of shrimp paste is providing a salty and umami taste, resulting from enzymatic protein hydrolysis to produce high amino acid content and nucleotides (Hajeb & Jinap, 2012). Therefore, shrimp paste is often used as an ingredient in various dishes to enhance the taste and flavor. The most famous and widely popular are shrimp paste chili sauce (Nam Prick Kapi), spicy shrimp paste soup (Kaeng Lieng), shrimp paste fried rice (Khao Kluk Kapi) and sweet-shrimp paste dip (Kapi Wan). At present, shrimp paste products are developed to match the consumer's lifestyles by generation, for example, low sodium shrimp paste (Cai et al., 2017; Jittrepotch et al., 2020; Rujirapong et al., 2022), shrimp paste powder (Sari et al., 2018), shrimp paste crackers (Retnaningsih et al., 2021) and instant shrimp paste chili sauce powder (Intarapan et al., 2021). However, the products are not versatile and little research has been reported.

The sweet-shrimp paste dip named Kapi wan refers to the ready-to-eat product that contains as its main ingredients sugar and shrimp paste and is prepared by simmering until sticky, then adding ingredients such as dried shrimp, dried fish, chopped onion and chili (TCPS 1496/2561). It's a Thai traditional fruit dipping sauce, however a similar recipe can be found in Philipines (Ginisang Bagoong), Vietnam (Mắm Ruốc Tôm Chấm Xoài), Malaysia, Indonesia and Singapore (Rojak) (Liagre, 2020; Pham, 2021; Manalo, 2022). Generally, this product has the characteristic of sticky liquid and a strong aroma which takes a long time to prepare. Kapi wan is usually sold in plastic packaging or glass jars. The glass jars are very heavy and inconvenient to move and transport. Moreover, a long storage time in unsuitable conditions will cause the product to deteriorate. According to the limitations, developing the sweet-shrimp paste dip into an instant product is a challenge. The product form of an instant dried powder offers the advantage of smell reduction, prolonged shelf-life, lightweight and easy to carry. Interestingly, it is possible to expand the export market as well, especially for the Asian population of overseas as well as offering convenience to customer who wants to save time in the kitchen.

Thus, this research studied the proportion ratio of ingredients (shrimp paste powder, palm sugar powder and cayenne pepper) in order to develop an instant sweet-shrimp paste powder using a mixture design experiment. The qualities of the finished product in terms of physical, chemical and sensory characteristics were investigated.

Materials and methods

1. Raw material and ingredients

The completely ripened shrimp paste (Ruea Thai, Kra Sah Khao Community Enterprise, Samut Sakorn, Thailand), palm sugar powder (Dao Rai, Community Enterprise of Ban Khlong Chaun Housewives Farmers Group, Songkla, Thailand), cayenne pepper (Rai Thip, Siam Makro Public Company Limited, Thailand) and dried shrimp (Worarat market, Sathorn, Bangkok, Thailand) were used in the experiment. Before experimenting, the dried shrimp was grounded into a tiny flake using a blender.

2. Shrimp-paste powder preparation

Shrimp paste was molded into a ball with a diameter of 1.5 cm. and placed in an aluminum tray followed by sun-dried until the moisture content reduced to less than 40% (7 hr). This process was conducted on a sunny day with a climate around 32-35°C from January to March 2020. To evaluate the moisture content, three pieces of a ball were picked randomly for examination using the infrared moisture balance (FD 720; Kett, Japan) every 1-2 hr. The of drying was performed by grounding the shrimp paste ball into a small piece and placed in the hot air oven at 70°C for 5 hr. After that, the sample was blended to obtain a fine powder (8.53% moisture content) using a blender (MX-AC400, Panasonic, Thailand). The preparation method was derived from the preliminary experiment, which found that the partially dried with the sun significantly preserved the shrimp-paste flavor and color better than when the whole process used a hot air oven.

3. Experimental Design

The mixture design was used to formulate the combination of three components, *e.g.,* shrimp paste

powder (40-60%), palm sugar powder (10-30%) and cayenne pepper (10-30%). The fixed factor was 30% ground-dried shrimp. The studied points in the corresponding triangle area were determined using the Minitab V. 17 programs, including seven treatments as shown in Table 1. Table 2 presents the percentage of an ingredient in each treatment when mixed with ground-dried shrimp.

The optimal treatment was selected by the overlapping contour plot of 5 characteristics of a sensory attribute (appearance, color, flavor, taste and overall liking), described in sub-topic 7.

Table 1 The weight of the ingredients of sweet-shrimp paste powder in different treatments

Ingredient (g)	Treatment*						
	1	2	3	4	5	6	7
Shrimp paste powder	60.00	56.67	56.67	60.00	53.34	46.66	40.00
Palm sugar powder	30.00	26.67	16.67	10.00	23.33	26.67	30.00
Cayenne pepper	10.00	16.66	26.66	30.00	23.33	26.67	30.00

Remark: * All treatments were mixed with the 30 g of ground dried shrimp to obtain 130 g total weight

Table 2 Percentage of the ingredients of sweet-shrimp paste powder in different treatments

Ingredient (g)	Treatment*						
	1	2	3	4	5	6	7
Shrimp paste powder	46.15	43.59	43.59	46.15	41.03	35.89	30.77
Palm sugar powder	23.08	20.52	12.82	7.69	17.95	20.52	23.08
Cayenne pepper	7.69	12.82	20.51	23.08	17.95	20.52	23.08
Ground dried shrimp	23.08	23.08	23.08	23.08	23.08	23.08	23.08
Total	100	100	100	100	100	100	100

Remark: * All treatments were mixed with the 30 g of ground dried shrimp to obtain 130 g total weight

4. Moisture content and water activity (a_w)

The moisture content of sweet-shrimp paste powder was analyzed according to the standard method of AOAC (2000) using a hot air oven at the condition of 105°C. The a_w was measured using the water activity meter (Serie 3TE, Aqualab, Switzerland) and calibrated with distilled water to obtain the a_w of 1.00±0.01 before the experiment.

5. Color

The color of sweet-shrimp paste powder was measured using a color meter (D/8-S, Miniscan XE Plus, USA) and reported in the Hunter Lab system (L*, a*, b*). The L* referred to lightness in which 0 = black and 100=white. The a* referred to redness (positive value) and greenness (negative value). The b* referred to yellowness (positive value) and blueness (negative value).

6. Protein, fat, carbohydrate and ash contents

Protein, fat, carbohydrate and ash contents were determined according to the standard method of AOAC (2000). Crude protein was analyzed using the Kjeldahl method based on the total nitrogen in the sample. The percentage of crude protein was reported by multiplying the determined total nitrogen by a nitrogen-to-protein conversion factor (6.25). Crude fat was analyzed by the Soxhlet method, using petroleum ether as the solvent extraction. For ash analysis, the defatted sample was incinerated in the furnace at 535°C overnight until obtaining a white color with stable weight. Total carbohydrate content was determined by calculating the percent remaining after all other components has been subtracted.

7. Sensory evaluation

Sensory attributes of seven formulas of sweet-shrimp paste powder in terms of appearance, color, flavor, taste and overall liking were evaluated by 30 untrained panelists. All samples were individually coded with a 3-digit number. To prepare samples, the panelist was asked to pour one part of warm water into two parts of a mixture of dry powder that was placed in a white ceramic cup, followed by stirring to give a continuous paste (around 30-45sec). The sample was served with a piece of green mango (*cv.* Khiew Sawoey). The panelists were suggested to score in five characteristics of the sensory attributes using the 9-point hedonic scale (1= dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5= neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much and 9=like extremely). An appearance was defined as the size, shape of dried-mix powder and the consistency of rehydrated product. A visual color appearance and a flavor sensation perceived by the test organ are characteristic descriptions of color and flavor, respectively. For the characteristic of taste, a preference for sweet, sour, salt and umami perceived by the tongue was noted. The whole satisfaction of the products was scored in terms of overall liking. Furthermore, clearing the mouth with drinking water was requested for all panelists before testing the next sample.

8. Statistical analysis

The experiment was conducted using a mixture design including 7 treatment combinations. All analyses were performed in triplicate and reported as mean ± standard deviation. Comparison of the mean value by Duncan's multiple range test for seven treatments and Independent sample t test for two treatments, using SPSS program V.

27 (An IBM Company, Ontario, Canada) for analysis of variance at $P \leq 0.05$. The contour and overlay graph were created using Minitab Program V.17 (Minitab, LLC., USA).

Results and discussion

1. Physical properties of shrimp paste powder

The physical properties of shrimp pasted powder prepared by partial reduction of moisture content by the sun before drying in a hot air oven are shown in Table 3. The moisture content of the sample was lower than 10%, according to the criteria of the Thai Community Products Standard of shrimp paste powder (TCPS 675/2561). The percentage of moisture content was a direct correlation to the low a_w value, shown in Table 3 as being 0.47. The criteria a_w did not exceeded 0.85 (TCPS 675/2561). The visible color of shrimp paste powder was purple-brown as the natural color of raw material, correlating to the low L* value (close to black; 0), positive a* (redness) and b* (yellowness). The drying operations could retain the color, flavor and aroma of raw material better than the application of hot air throughout the process (data not shown).

Table 3 Physical properties of shrimp paste powder

Parameter	Value
Moisture content (%)	8.53 ± 0.57
Water activity (a_w)	0.47 ± 0.01
L*	32.07±5.71
a*	5.54 ± 0.36
b*	9.64±1.36

Remark: The data presented by mean ± SD (n = 3)

To prepare shrimp paste powder, it is necessary to remove partial moisture content by sun-drying before the second step of dehydration in a hot air oven to preserve the flavor and color of the product. The total time for drying was 12 hr (7 h; 1st step and 5 h; 2nd step). The development of natural brown color in shrimp paste occurred through the enzymatic and non-enzymatic processes (Daroonpunt et al., 2016). Free amino acids and small peptides could undergo a Maillard reaction during fermentation, contributing to a non-enzymatic browning (Pongsetkul et al., 2019). For enzymatic browning, it is principally caused by the melanosis induced by the polyphenol oxidase (PPO)-catalyzed oxidation of tyrosine and its derivatives. The shrimp PPO is commonly known as tyrosinase, which is related to the biosynthesis of melanin (Shao et al., 2019). However, the prolonged hot air drying resulted in fading of dried-powder color (higher L* and b* value) observed in the preliminary experiment (data not shown), possibly due to the oxidation of free astaxanthin under the condition accordingly (Daroonpunt et al., 2016). This observation was in agreement with Sari et al. (2018), the low drying temperature (40°C, 6 days) of dried shrimp paste mixed with Angkak pigment could retain the chemical composition and antioxidant activity effectively when compared to a high temperature (50 and 60°C). The flavor volatile substance might be destroyed during the dehydration process, *e.g.,* Trimethylamine, 2,5- Dimethylpyrazine, 2,6-Dimethylpyrazine, 2,5-Dimethyl-3-Ethylpyrazine, 2-Ethyl-5-Methylpyrazine, 2-Methylbutyric Acid, 3-Methyl Butanal, 3-Methylbutanol and Dimethyl Trisulfide (Kleekayai et al., 2016).

Shrimp paste is a good source of polyunsaturated fatty acid (PUFA), such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA), which have many beneficial effects on human health (Pongsetkul et al., 2019). These PUFA has been reported to lowering plasma cholesterol (Zuliani et al., 2009), the prevalence of cardiovascular diseases and acute cardiac death (Ander et al., 2003). Noticeably, the development of flavor in shrimp paste occurred during periods of fermentation process undergo lipolysis by endogenous and microbial enzymes in raw material and further oxidation. Aldehyde and ketone are outstandingly oxidation products obtained from the oxidation processes (Pongsetkul et al., 2017; Pongsetkul et al., 2019). However, a higher rate of lipid oxidation might limit its shelf-life because oxidation products are generally further decomposed to off-flavor volatiles (Pongsetkul et al., 2022). According to the effect of heat, it might destroy the flavor and aroma compound, which is considered as a natural characteristic of shrimp paste. It is also experienced in the same way as an important fatty acid related to health promotion. The effect of heat or processing conditions has been reported in related products such as salts shrimp powder (AlFaris et al., 2022) and foam-mat dried shrimp powder (Azizpour et al., 2016). The preparation of salted shrimp by grilling (15 min), frying (7 min), or boiling (15 min), before subsequently drying at 50°C for 72 hr and further crushing into a powder, affected their nutritional and antioxidant profiles, especially grilling (AlFaris et al., 2022). The foam-mat dried shrimp powder exposed to the temperature of 90°C resulted in the development of

a Maillard reaction when compared to 40, 60 and 75°C, indicated by increasing a* value and declining L* value (Azizpour et al., 2016).

A different technique to prepare shrimp paste powder was reported by Barcelon et al. (2023), in which shrimp paste was diluted with water (1:1 to 1:4) and further undergoes spray drying. The factors that affected yield, moisture content and a_w value were temperature inlet (140-180°C) and feed flowrate (15-35 mL/min), while the low dilution ratio (1:1 and 1:2) affected better consumer acceptability in color, aroma and flavor.

2. Selecting the optimal formula of sweet-shrimp paste powder

The dried shrimp paste was used as the main ingredient of three components (shrimp paste powder, palm sugar powder and cayenne pepper) to produce an instant sweet shrimp paste powder. Three components of the mixture contour plot of qualities factor of appearance, color, flavor, taste and overall liking are shown in Fig. 1 (a-e). The results showed that an increase in palm sugar tends to raise sensory attributes of appearance, flavor, taste and overall liking (Fig. 1a, c, d and e). A large amount of cayenne pepper experienced a higher score of color (Fig. 1b). Nevertheless, the appearance and taste characteristics declined when the proportion of cayenne pepper increased (Fig. 1a and c). The sensory characteristics in terms of appearance, color, flavor, taste and overall liking of seven treatment combinations were in the range of 6.33-6.50, 6.07-6.43, 6.13-6.70, 5.77-6.53 and 6.07-6.53, respectively, however, there was no significant difference ($p>0.05$) between treatments (Table 4). Thus, the overlapping area of the contour plot was used to select the best formula given the appropriate concentration of each factor.

The overlapping graph of all sensory attributes of sweet-shrimp paste powder is shown in Fig. 2. The area of treatment No. 1 (60% shrimp paste powder, 30% palm sugar powder and 10% cayenne pepper) and 5 (53.34% shrimp paste powder, 23.33% palm

Table 4 Sensory score of seven treatments of sweet-shrimp paste powder

Sensory attributes	Treatment						
	1	2	3	4	5	6	7
Appearance [ns]	6.37±1.25	6.50±1.22	6.33±1.27	6.33±1.47	6.47±1.38	6.43±1.41	6.50±1.38
Color [ns]	6.07±1.36	6.17±1.23	6.40±1.35	6.40±1.50	6.43±1.22	6.40±1.40	6.40±1.35
Flavor [ns]	6.47±1.38	6.70±1.15	6.40±1.35	6.60±1.52	6.20±1.37	6.40±1.48	6.13±1.33
Taste [ns]	6.53±1.68	6.33±1.24	5.77±1.55	6.03±1.43	6.17±1.46	6.50±1.28	6.23±1.28
Overall liking [ns]	6.53±1.43	6.33±1.15	6.07±1.41	.10±1.30	6.33±1.30	6.43±1.36	6.17±1.42

Remark: ns = no significant difference

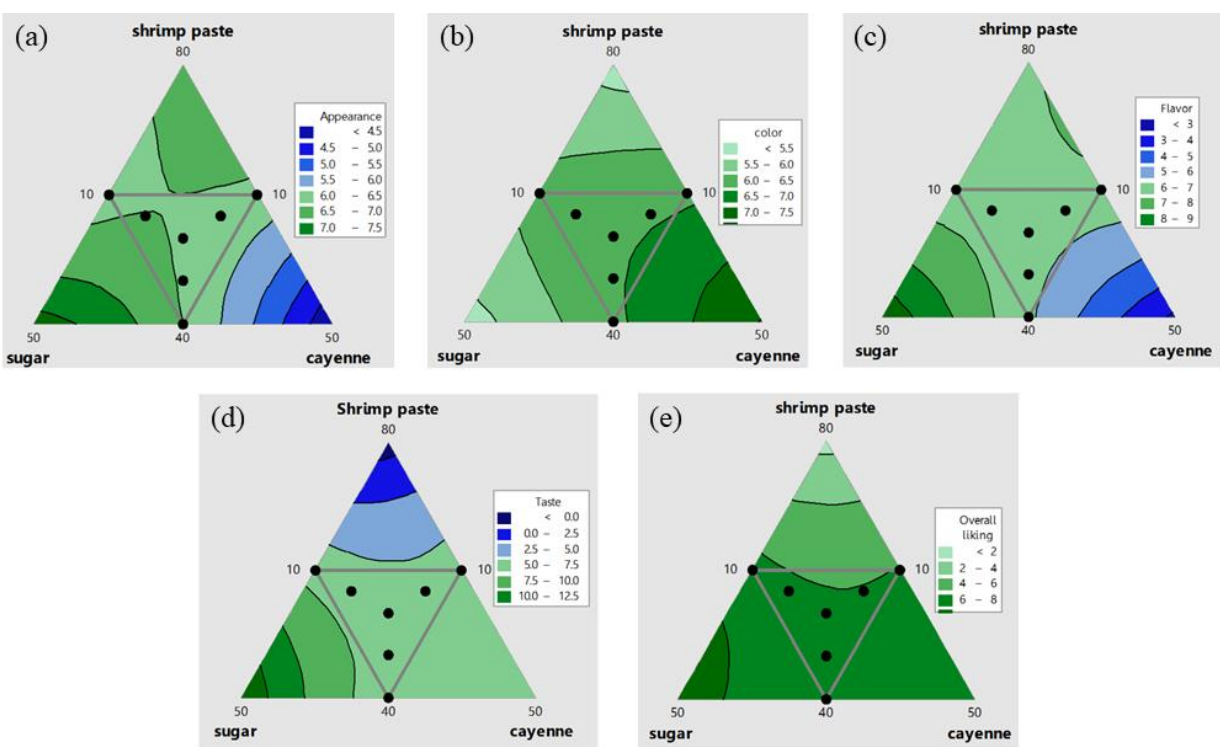

Fig. 1 Mixture contour plot of three compositions for the sensory attributes: appearance (a), color (b), flavor (c), taste (d), and overall liking (e) of sweet-shrimp paste powder

sugar powder and 23.33% cayenne pepper) showed the most overlapping point for all considered factors, confirmed by the highest score of taste (6.53±1.68) and overall liking (6.53±1.43), even though it was not significantly different (p>0.05) from other treatments (Table 4). For treatment No. 5, the good appearance (6.47±1.38) and color (6.43±1.22) were noted, while the overall liking score was 6.33±1.30. All sensory attributes of both treatments ranged from like slightly to like moderately. The appearance of treatment No. 5 had a brighter red color than treatment No. 1 because of a high proportion of cayenne pepper (Figure 3), indicated by a high a* value (Table 5). Both treatments contained similar ingredient content, except for cayenne pepper (low and high levels), indicating the panelists separating into like and dislike spicy.

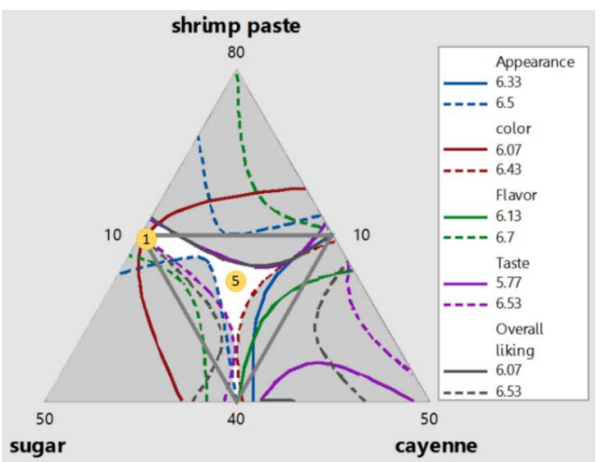

Fig. 2 Overlapping graph of all sensory attributes, selected from the area with the appearance score between 6.33-6.5, color 6.07-6.43, flavor 6.13-6.70, taste 5.77-6.53 and overall liking 6.07-6.53. The area marked with No. 1 represented the formulation of 60% shrimp paste powder, 30% palm sugar powder and 10% cayenne pepper. The area marked with No. 5 represented the formulation of 53.34% shrimp paste powder, 23.33% palm sugar powder and 23.33% cayenne pepper

Fig. 3 Appearance of all treatments of an instant sweet-shrimp paste powder

3. Physicochemical properties of sweet-shrimp paste powder

The sweet-shrimp paste powder in treatments 1 and 5 were selected for determining the physicochemical properties in terms of color (L*, a* and b*) and proximate components as shown in Table 5. The percentage of moisture content of the sample in treatment No. 5 (13.99%) was significantly higher (p≤0.05) than in treatment No. 1 (12.01%), which was in the same trend of a_w value, shown in Table 5 as being 0.52 (treatment No. 1) and 0.53 (treatment No. 5). However, there was no significant difference (p>0.05) in a_w value between treatments. Presently, there are no Thai standard for the sweet-shrimp paste powder or other related products, however, the moisture content of this product was higher than the standard of shrimp paste powder which did not exceed 10% (TCPS 675/2561). This might be due to it containing other ingredients, especially a palm sugar powder, identified as a hygroscopic material that acted to absorb moisture quickly under high relative humidity (Saputro et al., 2020). For a suggestion, the roasting could be applied to a final product to eliminate some moisture content of dried mix for prolonging the shelf-life.

The appearance of treatment No. 5 was visually red (Fig. 3) resulting in a higher a* value (11.59) compared to treatment No. 1, because of the high content of cayenne pepper. For chemical properties, the content of protein (32.73-33.96%) and carbohydrate (30.60-31.49%) in both treatments were similar, which had no statistically significant (p>0.05) difference between treatments. However, a large amount of protein content was observed in treatment No. 1, because it was rich in protein sources, including shrimp-paste powder (46.15%) and ground dried shrimp (23.07%), as shown in Table 2. The fat content of treatment No. 5 (4.73%) was significantly higher (p≤0.05) than treatment No. 1 (3.86%), which might be due to it containing a large amount of cayenne pepper (17.95%). The nutritional labeling of raw material of cayenne pepper showed 17g/100g total fat content (26% of daily value). The standard of total fat content in cayenne pepper reported by the U.S. Department of Agriculture is 17.3g/100g (USDA, 2019), considered to be similar to the raw material used in this experiment. The ash content in food refers to the mineral or inorganic remaining after removing moisture, volatiles and organic matter by complete oxidation under a very high temperature. Treatment No. 1 consisted of a higher ratio of shrimp paste powder (46.15%) which was

abundant in $CaCO_3$ in whole shrimp containing shell supplemented with NaCl as a preservative (Pongsetkul et al., 2015). Moreover, dried shrimp is a source of calcium, potassium, copper, iron, phosphorus, magnesium, zinc and sodium (AlFaris et al., 2022).

Table 5 Physicochemical properties of sweet-shrimp paste powder

Parameter	Treatment	
	1	5
Moisture content (%)	12.01 ± 0.18^{b}	13.99 ± 1.33^{a}
Water activity (a_w)	0.52 ± 0.00^{ns}	0.53 ± 0.01^{ns}
L*	15.81 ± 1.46^{ns}	16.96 ± 2.68^{ns}
a*	9.79 ± 0.47^{ns}	11.59 ± 1.06^{ns}
b*	16.32 ± 0.66^{ns}	18.07 ± 2.47^{ns}
Protein (%)	33.52 ± 0.38^{ns}	32.31 ± 0.99^{ns}
Fat (%)	3.86 ± 0.02^{b}	4.73 ± 0.09^{a}
Carbohydrate (%)	31.49 ± 0.41^{ns}	30.60 ± 0.66^{ns}
Ash (%)	19.01 ± 0.57^{a}	18.37 ± 0.10^{b}

Remark: The data is presented by mean ± SD (n = 3)
The value marked by different letters represented the significant difference ($p \leq 0.05$). ns = not significant difference

Sweet-shrimp paste powder is a new product as an instant dipping sauce for fruits that provides sweet, savory and umami taste. The enlargement of the umami taste occurred during the fermentation and formed a degraded product, amino acid, nucleotides and salt (Hajeb & Jinap, 2012). There has been a report that glutamic and aspartic content in Kapi was 3.96-4.3 and 2.24-2.55 mg/100g, which were the main features of taste attributes (Kleekayai et al., 2016). This is the first time reporting on the development of instant sweet-shrimp paste powder. However, a similar product of instant Nam Prick Kapi (Thai shrimp paste chili sauce) powder has been noted by Intarapan et al. (2021), prepared by tray drying at 70°C for 6 h. The instant Nam Prick Kapi powder had a 9.10% moisture content and the a_w value was 0.37. In comparison to this study, the a_w value of sweet-shrimp paste powder was around 0.52, characterized as a dried food (a_w <0.6), which inhibited the microorganism growth (Fellows, 2009).

Conclusion

The best treatment for producing an instant sweet-shrimp paste powder was treatment No. 1, containing the ingredients of 46.15% shrimp paste powder, 23.08% palm sugar powder, 7.69% cayenne pepper and 23.07% ground dried shrimp. All sensory attributes were classified as good (ranging from like slightly to like moderately), especially in terms of taste and overall liking, which received better scores than other characteristics. The finished product had a 12.01% moisture content and the a_w value was 0.52. The percentage of crude protein, fat, carbohydrate and ash content were 33.52, 3.86, 31.48 and 19.01%, respectively. Treatment No. 5 was recommended alternatively for producing the spicy formula as it contains a large amount of cayenne pepper. The results of this study showed the processing was suitable for manufacturing an instant sweet-shrimp paste powder for small and medium enterprises (SMEs). The consumption could be directly dipped or filled with warm water as a semi-instant product. Further study should be conducted to consider microorganism safety and the shelf-life of the product. For commercial applications, unique features such as a rehydration ratio or viscosity, including a consumer test with a larger population (at least 50 participants) of two selected formulas should be investigated. The product should be kept in a package preventing moisture absorption during storage periods to preserve the product quality.

Acknowledgment

The authors offer appreciation to the Faculty of Science and Technology, Rajamangala University of Technology Krungthep for supporting all facets of this research.

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Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Antioxidant, Antidiabetic and Antiproliferative Activities of Juice Extract and Residue Powder from Karanda (*Carissa carandas* L.)

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Article info

Article history:
Received : 5 February 2023
Revised : 29 March 2023
Accepted : 7 April 2023

Keywords:
Carissa carandas, Antidiabetes, Antiproliferate, Freeze dried, Karanda powder

Abstract

Karanda (*Carissa carandas* L.) fruit has been extensively studied for various applications and used in making a variety of food and nonfood products due to the phytochemical components. In this study, the phenolic content, antioxidant activity, anti-diabetes and antiproliferative activities of the freeze-dried crude karanda juice powder (KJP) and residue powder (KRP) were investigated. The findings revealed a different yield: KJP obtained 7.76 g/100g and KRP obtained 9.89 g/100g ($p \leq 0.05$). In addition, the KRP had less moisture and water activity (a_w) than the KJP, which may be associated with the sugar residue content. The KJP revealed that the total phenolic and anthocyanin content were 58.02 mg GAE/g and 0.17 mg/g, respectively. Furthermore, antioxidation activity using the DPPH and FRAP assays demonstrated 38.86 mg GAE/g and 9.08 µmol $FeSO_4$/g, respectively. In terms of anti-diabetes activity, the crude extract of KJP inhibited α-amylase activity (98.70%) and α-glucosidase (50.86%), whereas KRP inhibited α-amylase activity (50.34%) and α-glucosidase (18.30%) when compared to acarbose. The KRP and KJP were both non-cytotoxic and inhibited the growth of human colorectal adenocarcinoma (Caco-2) cells and human liver hepatocellular carcinoma (HepG2), with IC_{50} values of 97.26 µg/mL and 87.06 µg/mL, respectively. Both the KJP and the KRP freeze-dried showed promise as dietary supplements or functional foods for diabetes and cancer prevention.

Introduction

Carissa carandas L. is a member of the Apocynaceae family that is commonly grown in Malaysia, Thailand, India, Philippines, Cambodia, Vietnam and East Africa. It is known to possess an extensive range of phytochemicals in its fruits that impact enormous medicinal value including vitamin C, anthocyanin, flavonoids, glycosides, alkaloids, carbohydrates, sterols, terpenoids, tannins and saponins

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(Monika et al., 2022; Bhaskar & Balakrishnan, 2009). The unripe fruit has abundant quantities of ferulic acid (80.04 mg/g) and sinapic acid (16.29 mg/g) (Kubola, et al., 2011). These active compounds have medical and pharmacological value as anti-inflammatory, antipyretic, antioxidant, anti-cancer and antidiabetic and antiviral property. (Iyer & Dubash, 2006; Begum et al., 2013; Sudjaroen & Suwannahong, 2017).

Particularly, the fully ripened fruit turns a purple color and contains anthocyanins such as cyanidin-3-o-rhamnoglucoside (Iyer & Dubash, 2006), cyanidin-3-galactoside, cyanidin-3-glucoside (Rohan et al., 2018) and simple phenolics such as hydrocinnamic acid. Anthocyanins, which are red, purple and blue in color, have been classified as powerful functional foods with great potential as food colorants because of their high pigment content and low toxicity, which have increased interest in their development to replace synthetic colorants (Brouillard, 1982). Anthocyanins are used as food coloring in confections, jellies, jams, preserves and frozen desserts, among others (Birks, 1999). Numerous studies have documented the health benefits of anthocyanins (Chen et al., 2005; Wang & Stoner, 2008). Antioxidants are essential substances that have the ability to protect the human body from free radical damage (Madhuri et al., 2019). Antioxidants can help prevent the formation of free radicals by scavenging them or promoting their decomposition and suppression.

Previously, karanda juice was extracted and processed as a functional beverage, jelly and pudding. The remaining fruit, seed and peel after juice extraction were not used. To maximize the value of the source material and reduce industrial waste, these residues might be employed as dietary supplements or functional foods. Freeze-drying is commonly employed for the long-term storage and preservation of foods. This is one of the most exciting and useful procedures for drying food since it protects and retains the characteristics of fresh samples, such as shape, appearance, flavor, nutrients, porosity, color, flavor, texture and biological activity. Consequently, the goals of this study were to evaluate the chemical and biological analysis of fruit extracts and residue powder from *C. carandas* that can be further used as functional foods.

Materials and methods

1. Sample collection and preparation

Fully ripened dark purple to black karanda (*Carissa carandas* L.) fruits were harvested from Tha Maka Kanchanaburi Province, Thailand between July to August 2021. One kilogram of fruits was washed with water and soaked in water for 10 min. The fruits were then placed in a plastic basket and rinsed under running tap water for 5 min. Next, the fruits were separated by juice extractor (Panasonic MJ-DJ01, Japan) into juice extract and residue. The juice extract was concentrated again using a rotary evaporator (BUCHI R300, Japan) at 60°C until 18°Brix solid and then freeze-dried (EPSILON FD 60, Thailand) as juice extract powder (KJP). The residue (containing seed and pulp) after extraction (KRP) was directly pre-chilled at -20°C before freeze drying. The freeze drying process consisted of 1) pre-freezing process at -25°C for 180 min, 2) primary drying at -15°C to 35°C for 480 min and 3) secondary drying at 45°C to 50°C for 30 to 60 min. After that, samples (500 g) were ground into a fine powder using a dry powder herb grinder with high blade rotation at 25000-32000 rpm, 2500 watts and 220 volts. Next, the samples were sifted through a 100 mesh stainless steel sieve and packed into aluminum foil bags for analysis.

2. Determination of yield, physical and chemical characteristics

The karanda powder was calculated by weighing the fruit, freezing-drying fruit extract and residue and then measuring the net weight and percentage of the by-product. After that, the color of the KJP and KRP were measured using a colorimeter (Minolta Colorimeter, CR 400, Japan) in the CIE system (L*a*b*). The pH values were determined by a pH meter (Mettler-Toledo, USA). Finally, the moisture content was determined following the AOAC method (AOAC, 2000). Water activity (a_w) was measured using a water activity meter (Smart Water Activity Meter model AQUALAB 4 TE, USA).

3. Preparation and analysis of crude extract from KJP and KRP

One gram of sample was soaked in 50 mL of 40% ethanol in water and then shaken in an ultrasonic bath (Wiggens UE03SFD, Germany) at 55°C for 60 min. The extract was filtered through No. 4 Whatman filter paper.

3.1 Determination of total phenolic content using by Folin-Ciocalteu reagent

Total phenolic content was determined by the Folin-Ciocalteu reagent following Gorinstein et al., (2001) with slight modifications. First, 0.75 mL of 10-fold diluted Folin-Ciocalteu reagent and 100 mL of the ethanolic extract were placed in a test tube. The mixture

was mixed and allowed to stand at room temperature for 5 min. Then, 0.75 mL of a 6% (w/v) sodium carbonate solution was added. The mixture was homogenized and allowed to stand at room temperature for 90 min.Total phenolic content was determined at 725 nm using a spectrophotometer (Thermo scienctific Evaluation 201, USA). A standard calibration curve was plotted using ascorbic acid at a concentration of 0.02-0.1 mg/mL. Total phenolic content was expressed as gallic acid equivalent (GAE) mg/g (Pewlong et al., 2014).

3.2 Determination of total anthocyanin content using the pH-differential spectrophotometric method

Each sample was diluted with two dilutions including potassium chloride (0.025 M) at pH 1.0 and sodium acetate (0.4 M) at pH 4.5. Samples were allowed to equilibrate for 15 min before detection by a spectrophotometer (Thermo scienctific Evaluation 201, USA), with absorbance measured at 520 nm ($A_{520\,nm}$) and 700 nm ($A_{700\,nm}$). The difference in the absorbance at differing pH values and wavelengths was calculated as:

$$A = (A_{520\,nm} - A_{700\,nm})pH1.0 - (A_{520\,nm} - A_{700\,nm})\ pH4.5$$

The concentration of total anthocyanin pigments was calculated as:

$$\text{Total anthocyanin content (mg/L)} = (A \times MW \times DF \times 1000)/\varepsilon x1$$

Where MW is the molecular weight, DF is the dilution factor, ε is the molar absorptivity and 1 is for 1 cm path length. The molecular weight (MW = 449.2 g mol^{-1}) and molar absorptivity (ε = 26,900 Lcm^{-1} mol^{-1}) of cyanidin-3-glucoside were used (Wrolstad et al., 2005).

3.3 Antioxidant assays; DPPH radical scavenging activity

The DPPH radical scavenging activity was performed as previously described by Khattak et al. (2008). A measurement of antioxidant were 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. One hundred microliter of each extract sample was added to 900 µL of DPPH in method solution (150 µM) in a test tube and shaken vigorously. After incubation at room temperature (25°C) for 15 min in darkness, the absorbance of sample solution was determined at 517 nm. The free radical scavenging activity was expressed as mg ascorbic acid equivalent from 0.01 to 0.1 mg/mL, calculated by standard curve (R^2 = 0.9925).

3.4 Antioxidant assays; Ferric reduction antioxidant potential (FRAP)

Ferric reduction antioxidant potential of KJP and KRP were determined according to the method described by Benzei & Strain. (1996). The reaction reduced the ferric ion (Fe_3^+) to the ferrous ion (Fe_2^+) that changed the color of yellow complex (Fe_3^+/TPTZ) to blue complex (Fe_2^+/TPTZ). For analysis the FRAP reagent was prepared by mixing 16.7 mM $FeCl_3.6H_2O$ and 8.3 mM 2,4,6-tripyridyl-s-striazine (TPTZ) with 250 mM acetate buffer, pH 3.6. A total reaction of 5.25 mL consist of 75 µL sample, 225 µL of distilled water and added to 2.25 mL of freshly prepared FRAP reagent in a test tube. The solution was incubated at room temperature (25°C) throughout the reaction. The absorbance was read at 596 nm using a spectrophotometer (Thermo Scienctific Evaluation 201, USA) immediately and 30 min after mixing. The antioxidant potential of the samples was analyzed based on a standard curve plotted using $FeSO_4.7H_2O$ at different concentrations from 400 to 2,000 µM. The amount of antioxidant potential was expressed in µmol$FeSO_4$/g.

4. Antidiabetic activity assay

Crude extract and residue powder were extracted before determining α-amylase and α-glucosidase inhibition. Karanda extracts were prepared by aqueous extraction of 50 g of powder stirred in 200 mL of distilled water and 95% ethanol solution. The mixture was placed in a rotary shaker for 4 and 24 hr, followed by centrifugation at 8000 rpm for 10 min. The resultant supernatant was filtered using Whatman No.4 filter paper.

4.1 α-Amylase inhibitory assay

The determination of α-amylase inhibition was carried out by quantifying the reducing sugar (glucose equivalent) liberated under the assay conditions. The enzyme inhibitory activity was expressed as a decrease in units of glucose liberate. A modified dinitrosalicylic acid (DNS) method was adopted to estimate the glucose equivalent (Bhutkar & Bhise, 2012). An aliquot 1 mL of the aqueous extract of the selected karanda extracts was pre-incubated with α-amylase 1 unit/mL for 30 min and then 1 mL (1% w/v) starch solution was added. The mixture was further incubated at 37°C for 10 min. The reaction was stopped by adding 1 mL DNS reagent (12.0 g of sodium potassium tartrate tetrahydrate in 8 mL of 2 M NaOH and 96 mM 3,5-dinitrosalicylic acid solution) and the contents were heated in a boiling water bath for 5 min. A blank was prepared without plant extracts and another without the amylase enzyme, replaced by equal

quantities of buffer (20 mM sodium phosphate buffer with 6.7 mM Sodium chloride, pH 6.9 at 20°C). The absorbance was measured at 540 nm. Reducing sugar released from the starch was estimated as glucose equivalent from a standard graph. Acarbose was used as the positive control as inhibitor. The extracts were diluted in a buffer to give final concentrations of 5 mg/mL, 7 mg/mL and 9 mg/mL. The anti-diabetic activity was determined by the inhibition of α-amylase, expressed as percentage inhibition and calculated by the following equations:

% Inhibition = (Glucose) test / (Glucose) control x 100

4.2 α-Glucosidase inhibitory assay

Inhibition of α-glucosidase activity was determined using the modified published method (Berna et al., 2012). One milligram of α-glucosidase (Sigma-Aldrich, USA) was dissolved in 100 mL of phosphate buffer (pH 6.8) containing 200 mg of bovine serum albumin (Merck, Germany). The reaction mixture consisting of 10 µL of sample at varying concentrations (0.52 to 33 µg/mL) was premixed with 490 µL phosphate buffer pH 6.8 and 250 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) (Sigma-Aldrich, USA) and preincubated at 37°C for 5 min. Then 250 µL α-glucosidase (0.15 unit/mL) was added and incubated at 37°C for 15 min. The reaction was terminated by addition of 2000 µL Na_2CO_3 200 mM. The α-glucosidase activity was determined at 400 nm on spectrophotometer UV-Vis (Shimadzu 265, Japan) by measuring the quantity of *p*-nitrophenol released from *p*-NPG. Acarbose was used as the positive control of α-glucosidase inhibitor.

5. Cytotoxicity and cell viability assay using MTT assay

The *in vitro* cytotoxicity evaluation was performed using cancerous cell lines and normal cell lines. Human colorectal adenocarcinoma (Caco-2) cells and human liver hepatocellular carcinoma (HepG2) cells, as well as normal human fibroblast (BJ) cells, were used to determine the cytotoxicity of the samples. The cancer cells were seeded in a 96-well plate at a density of 2 x 10^4 cells/well in complete medium. Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), which contains 10% v/v heat-inactivated fetal bovine serum (FBS) and 1% v/v penicillin-streptomycin. HepG2 cells were cultured in DMEM containing 10% v/v FBS and 1% v/v penicillin-streptomycin. The normal BJ cells were seeded in a 96-well plate at a density of 3 x 10^4 cells/well in minimum essential medium (MEM) supplemented with 10% v/v heat-inactivated fetal bovine serum and 1% v/v penicillin-streptomycin. All seeded cells were incubated at 37°C in a humidified air atmosphere containing 5% (v/v) CO_2. After seeding for 24 hr, the cells were washed with serum free medium and incubated with the samples at concentrations of 1, 5, 10, 20, 50, 100 and 200 µg/mL. The final concentration of dimethyl sulfoxide (DMSO) in the culture medium of each treatment group was 0.5% (v/v). Cells in the serum free medium containing 0.5% (v/v) DMSO were used as the control. After 24 hr, the cells were washed with phosphate buffered saline (PBS) before being incubated for 4 hrs with a solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (final concentration 0.5 mg/mL in PBS). Finally, the medium was removed and DMSO was added to each well for dissolving formazan crystals and the absorbance at 540 nm was measured using a microplate reader. The experiment was performed with four replicates (Weerawatanakorn & Pan, 2017). Phytochemical components of *Carissa carandas* and the inhibitory effects of fruit juice on inducible nitric oxide synthase and cyclooxygenase-2.

6. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD) of three replicates, with data analyzed using the SPSS statistical package (SPSS, version 26.0, Chicago, IL, USA). The data was analyzed using t-test to evaluated the differences in means between KJP and KRP. The *P*-value was used to determine the level of significance at $\alpha \leq 0.05$.

Results and discussion

1. Yield of Karanda powder, moisture content, water activity and color

The karanda juice powder (KJP) and karanda residue powder (KRP) were effectively freeze-dried into a powder. The color characterisation, mechanical characteristics, porosity and water content of the freeze-dried KJP and KRP were assessed through experimentation. Traditional methods, such as sun drying, tray drying and spray drying, resulted in fewer modifications to the active constituents. After freeze-drying, the yields of KRP and KJP were 9.89 and 7.76 g/100 g, respectively. 45% of the residue after extraction yielded fresh fruit, which was less than the amount of fruit extracted (55%). Due to the extract's high water and

acidic content, the freeze-drying procedure is more complex and susceptible to moisture absorption. The KRP had 5.85% moisture, but the KJP contained 12.96% because the acidity of the KJP was a charged material that bound to water and moisture in the environment, as shown in Table 1. The KRP and KJP had water activity (a_w) values of 0.26 and 0.66, respectively.

The CIE (Commission International de L'Eclairage) or L* a* b* values of the two samples were different ($p \leq 0.05$). The KRP had a higher L* value due to its dispersion, flesh, peel and seeds that made the color lighter. The KJP was a combination of karanda flesh and juice after extraction, with further evaporation at 60°C. This process turned the extracted juice brown due to the browning reaction, which darkened the freeze-dried powder. The a* value of the KRP was higher than that of the KJP, at 34.09 and 30.02, respectively. Significantly water-soluble flavonoid compounds, such as anthocyanins in Karanda fruit, gave the KRP purple and red colors that were unstable at high heat. Before being freeze-dried, the two samples were heated in different ways during the preparation process. This caused the anthocyanin in the KJP sample to change color. The b* of the KRP showed a higher yellow value than the KJP at 16.11 and 12.08, respectively. The KJP became dark-purple and darker than the KRP, which was light purple.

2. Determination of total phenolic and anthocyanin content

The total phenolic content analysis revealed that the freeze-dried KJP had a total phenolic content of 69.68 mgGAE/g, which was higher than the KRP total phenolic content of 58.02 mgGAE/g, indicating that the flesh of the karadas fruit had a high total phenolic content. The KJP used in the extraction contained a mixture of flesh and juice. However, the KRP was a combination of the seeds and fruit pulp, which have accumulated various minerals and the amount of some bioactive compounds less than the KJP. According to Table 1, the total anthocyanin content of KJP was 0.13 mg/g, while the KRP contained 0.17 mg/g ($p \leq 0.05$). Pewlong et al. (2014) discovered that the total phenolic content in whole ripe karanda fruit was 4.67 mgGAE/g, which was the highest when compared to immature fruit. The total anthocyanin content was highest when the fruit was ripe, until it turned dark purple to black. Moreover, the KJP contained higher total phenolic content than KRP ($p \leq 0.05$). The KJP had higher karanda juice concentration and flesh than the KRP that included seeds. The KJP and KRP had total phenolic content of 67.68 and 58.02 mgGAE/g, respectively. Results concurred with Pewlong et al. (2014).

The KRP had 0.17 mg/g of total anthocyanin, while the KJP had 0.13 mg/g ($p \leq 0.05$) (Table 1). Purple berries contain glucose-conjugated anthocyanin. Acylated and non-acylated anthocyanins exist. Glucose, lactose, lutinoes and rhamnose are non-acylated anthocyanins, while acylation of glucose and acids such as coumaric, caffeic, malic and acetic generates esterification at the 3rd or 5th positions (Ambika et al., 2015). Acid bonding facilitate anthocyanin stability, which disintegrates at 100°C in mild acid solvents of pH 1– 4. The KRP had more anthocyanin than the KJP after evaporation. The concentration process at 60°C for 1 hr resulted in anthocyanin release due to the long time taken for the thermal process.

Table 1 Physicochemical properties of KRP and KJP

Properties	KRP	KJP
Yield (g/100 g)	9.89±0.15[a]	7.76±0.05[b]
Moisture content (%)	5.58±0.35[b]	12.96±0.23[a]
a_w	0.26±0.01[b]	0.66±0.05[a]
Color		
L*	9.16±0.05[a]	6.23±0.07[b]
a*	34.09±0.18[a]	30.02±0.22[b]
b*	16.11±0.04[a]	12.08±0.44[b]
Total phenolic content (mgGAE/g)	58.02 ±0.95[b]	69.68 ±0.74[a]
Total anthocyanin content (mg/g)	0.17±0.31[a]	0.13±0.85[b]

Remark: Values are expressed as mean ± SD of triplicates. Different letters in the same row indicate statistically significant difference ($p \leq 0.05$)

3. Antioxidant activity of Karanda powder

The antioxidant effects attributed to the polyphenols of plants plays a pivotal role in protecting the body from oxidative stress, diabetes, cardiovascular diseases and cancer (Madhuri et al., 2019; Weerawatanakorn & Pan, 2017). Therefore, the plant-based therapeutics is a potential alternative therapy to explore due to their reported safety and nutraceutical benefits.

To compare the total phenolics and antioxidant capacity of the KJP and KRP. The TPC (Folin Caiocalteu), FRAP and DPPH were performed. Anthocyanins have antioxidant activity and effectively prevent DPPH free radical. The KRP and KJP prevented different DPPH free radicals ($p \leq 0.05$) at 38.86 and 33.65 mg AAE/g, respectively and was also found by Khunchalee & Charoenboon (2019) (Table 2). The FRAP assay of KRP and KJP at 9.08 and 9.84 (μmol$FeSO_4$/g).

Table 2 Antioxidant Activity (DPPH assay) and FRAP of KRP and KJP (1 mg/mL)

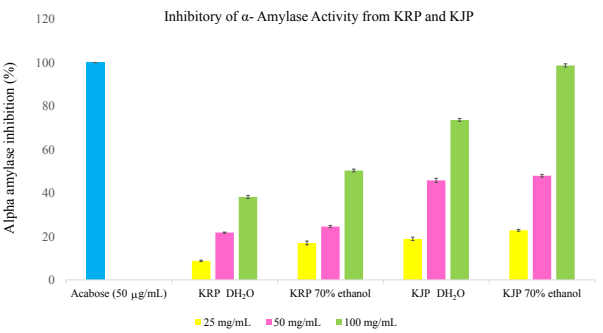
Antioxidant Activity	KRP	KJP
DPPH assay (mg AAE/g)	38.86±0.78[a]	33.65±0.43[b]
FRAP (μmol$FeSO_4$/g)[ns]	9.08±0.72	9.84±0.98

Remark: Values are expressed as mean ± SD of triplicates. Different letters in the same row indicate statistically significant differences ($p \leq 0.05$). ns = not significant ($p > 0.05$)

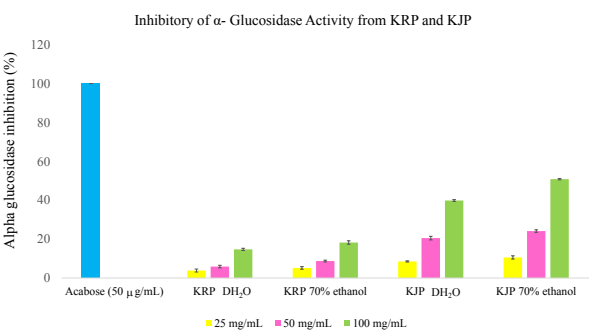
4. Assay for α-amylase and α-glucosidase Inhibition

Aside from antioxidant activity, numerous studies have shown that polyphenols have anti-hyperglycemic effects by binding to glucose transporters (Nistor et al., 2010) and competitively inhibiting digestive enzymes. Carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase, digest dietary starch and convert the oligosaccharides to glucose, resulting in an increase in blood glucose levels. As a result, one of the primary approaches to managing patients' hyperglycemic conditions is to inhibit these enzyme activities. Acarbose is the most commonly used α -glucosidase inhibitory drug, but it has side effects.

In this study, α-amylase and α-glucosidase inhibition assays were carried out at three different concentrations (25, 50 and 100 mg/mL) of water and ethanol extracts for 4 hrs and inhibition was observed at all concentrations (Fig.1). Acarbose (50 μg/mL) was used as a positive control. Fig.1 shows the inhibition of α-amylase and α-glucosidase activity by the tested KRP and KJP. The results showed that all the extracts possessed significant activity. The KJP at 100 mg/mL showed the highest activity at 98.70% (ethanol extract) and 73.62% (water extract), while the KRP has less inhibitory activity at 50.34% (ethanol extract) and 38.17% (water extract). Furthermore, the inhibitory values of ethanol and water extract for α-glucosidase inhibition were 50.86% and 39.89%, respectively. The KRP also revealed less inhibitory activity at 18.30% (ethanol extract) and 14.78% (water extract). As previously described, extracts containing 50 to 100 mg/mL of karandas have a higher potential to inhibit α-amylase than α-glucosidase. Ethanol extract exhibited the highest inhibition of both enzymes, which was significantly efficacious compared to acarbose. Several studies have suggested that plant extracts can act as α-glucosidase inhibitors, implying that these extracts may be useful in the treatment of hyperglycemia. The activity of these extracts, such as flavonoids, alkaloids, terpenoids, anthocyanins, glycosides and phenolic compounds, can be attributed to their phytoconstituent content. In the present study, among all the ethanol extracts, the KJP and KRP demonstrated a remarkable inhibition potential. The polyphenols are known to interact with the enzyme through non-specific binding, leading to inhibition of enzyme activity. Polyphenols become more effective at inhibiting α-glucosidase as their molecular weight and degree of polymerization increase (Wang et al., 2013).



(A)



(B)

Fig. 1 Inhibitory of α-Amylase Activity from karanda residue powder (KRP) and karanda juice powder (KJP) (A) and Inhibitory of α-Glucosidase Activity from karanda residue powder (KRP) and karanda juice powder (KJP) (B)

5. Cytotoxicity and cell viability assay

Polyphenols can inhibit the growth of a wide range of cancer cells. The sensitivity of cancer cells to polyphenol may differ depending on the tissue from which they are derived, implying a possible link between polyphenol-induced cytotoxicity and specific cancer types (Benavente-Garcia & Castillo, 2008). Here, anticancer activity of aqueous and ethanol extract and residue of *C. carandas* was studied by using MTT assay towards cell line including human fibroblast cells, HepG2 and Caco-2. Dose response curves constructed between 10-200 μg/mL ranges. The results showed that the

incubation of KJP with HepG2 and Caco-2 significantly inhibited cell proliferation with IC_{50} values of about 87.06 and 97.26 µg/mL, respectively. Growth inhibition percentage is illustrated and statistically ($p \leq 0.05$) in Table 3 The proliferation of HepG2 was significantly inhibited by 73.7% suppression while the proliferation of Caco-2 was significantly inhibited by 77.9% using the ethanol extract at 200 µg/mL.

Moreover, the KRP also showed the inhibition with IC_{50} values of about 13.28 mg/mL with HepG2 and 11.79 mg/mL with Caco-2. Our findings demonstrated that ethanol extract and residue may contain a high chemical component compared to previous reports in which a methanolic extract of *C. carandas* fruit was also found to have antiproliferative activity against MCF-7 and HepG2 cells (Pewlong et al., 2014; Priti et al., 2014; Ondee, 2019). Fruit juice containing major phenolic compounds such as ferulic acid, rutin, cyanidin-3-glucoside, flavonoids and anthocyanins has downregulated the induction of inflammatory responses. Polyphenolic compounds such as epicatechin gallate, quercetin, kaempferol and triterpenes, i.e. carissol. The cytotoxicity stopped the cell cycle and caused apoptosis. It also caused a big drop in superoxide anion production, cell adhesion and the movement of tumor cells (Ahamad et al., 2014).

The cytotoxic effect of the extract and residues showed that not only edible parts but also by-products exhibit a good source of anticarcinogenic agents. Therefore, karanda fruit powder showed promise and could be further developed as capsule or tablet supplements or functional foods such as antioxidant tea, antidiabetic tea, or tea for the prevention of cancer.

Table 3 Antiproliferative activities from KRP (mg/mL) and KJP (µg/mL)

Cells	Antiproliferative activities (IC_{50})		Reference
	KRP	KJP	
Fibroblast cell line	85.53±0.91[b]	130.97±0.67[a]	In this study
Caco-2 cell line	11.79±0.20[b]	97.26±0.53[a]	In this study
HepG-2 cell line	13.28±0.68[b]	87.06±0.73[a]	In this study
HepG-2 cell line		56.81±0.97	Priti et al., 2014
Hela cell line		58.62±0.35	Priti et al., 2014
MCF-7 cell line		56.72±0.59	Priti et al., 2014
MG-63 cell line		82.91±0.79	Priti et al., 2014

Remark: Values are expressed as mean ± SD of triplicates. Different letters in the same row indicate significance at $p \leq 0.05$

Conclusion

Our findings demonstrated that freeze-dried karanda powder is particularly beneficial for dietary supplements and functional foods. They have a range of phytochemical, anti-diabetic and anti-proliferative activities. KRP had less total phenolic content than KJP, but it had more total anthocyanin content and showed different antioxidant activity in the DPPH and FRAP assays ($p \leq 0.05$). They also demonstrated anti-diabetic activity by inhibiting α-amylase and α-glucosidase activities. KRP and KJP have less cytotoxicity but could suppress the growth of Caco-2 and HepG2 with IC_{50} values of 11.79 and 13.28 (mg/mL) and 97.26 and 87.06 (µg/mL), respectively. Based on their biological activity, these powders have the potential to be effective antioxidant supplements for diabetes and cancer prevention.

Acknowledgment

The authors acknowledge The Crocodile My Friend Co., Ltd. for the financial support under the research grant (Project No. GRANT1825).

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Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Process Development of Mozzarella Farm Cheese from Buffalo Milk

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Article info

Article history:
Received : 5 February 2023
Revised : 27 March 2023
Accepted : 10 April 2023

Keywords:
Mozzarella cheese, Buffalo milk, Cheese processing, Texture

Abstract

The objective of this research was to improve and develop the process of mozzarella cheese manufacturing from buffalo whole milk. Qualities of buffalo raw milk and manufacturing conditions were studied and included curd cutting time (0-75 min), cooking temperature (43, 45 or 47°C) and pre-stretched curd pH (5.1 and 4.9). The qualities of buffalo farm milk met the buffalo milk standards (TAS 6007-2021). A 10 kg pasteurized milk was inoculated with lactic acid bacteria at 40ºC and 0.1 g rennet was added after achieving 0.1 pH decrease in milk. Inoculation starter culture and rennet enzyme caused the pH to drop and milk coagulum to occur. The pH of cultured milk decreased with increasing hardness of milk curd after renneting. At 60 min after renneting the milk curd was suitable for cutting. Low cooking temperature (43°C) prevented cheese curds to coalesce. On the other hand, cooking the cheese curds at a higher temperature (47°C) promoted hardening of the cheese curds. While cooking temperature at 45°C the cheese curds were soft, agglomerated with good stretchability. Chemical compositions of cheese from pre-stretched curd pH 5.1 or 4.9 was similar. Textural quality in terms of hardness and chewiness of pre-stretched curd pH 5.1 were better than that of pH 4.9. The pre-stretched curd pH 5.1 had a better stretchability quality compared to pH 4.9. Recommended manufacturing conditions for mozzarella farm cheese were 60-min curd cutting time, 45°C cooking temperature and pre-stretched curd pH 5.1, respectively.

Introduction

Milk production and the processing industry play important roles in driving Thailand's economy. Thailand's dairy industry is growing along with advance in science and technology in terms of varieties, quality and safety of dairy products. Cow milk is the major raw material for the products, however insufficient raw milk production is a limitation for the development of Thailand's dairy industry. Dairy industry operators seek new sources of raw milk such as goat, sheep and buffalo milk, along with the development of processing to add value to new lactations, as a method to reduce importing dairy products. Especially cheese, which is in

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high demand in the food service industry. At present, local consumption of dairy products which includes foreigners residing in Thailand is increasing. Domestic cheese products are produced by large and small entrepreneurs. Mozzarella is the most popular cheese for the food service industry and related food processors such as pizza and bakery industry. Appropriate technology for producing mozzarella cheese helps small and medium enterprise (SME) to develop their cheese derived from the local milk supply.

Imported mozzarella cheese is sold in most markets in Thailand and is packed in brine as a soft fresh type of mozzarella cheese. Stretchability of heated mozzarella cheese is the most desired and accepted as a good cheese produced from buffalo milk. The texture of mozzarella cheese is influenced by manufacturing steps such as milk standardization, homogenization, cooking time and temperature, as well as pH of curds and stretching temperature. (Jana & Mandal, 2011; Paz et al., 2017). Cheese milk with high fat-to-protein ratio yields a softer cheese than that of higher protein milk. Low fat-to-protein ratio milk released some oil during the baking of the cheese (Guinee et al., 2000). Low-fat mozzarella cheese was harder in texture or springier than the full-fat cheese and showed poor melting characteristics (Tunick, 1991; Lefevere et al., 2000; Zisu & Shah, 2007).

Yazici et al. (2010) reported that curd pH at whey drainage was important to curd's ability to be laminated and stretched in hot water. While Paz et al. (2017) noted whey drainage at a low pH (around pH 5.0 - 5.3) which offered low hardness cheese with long and thin threads microstructure creating a distinctive texture of mozzarella cheese. In addition, structural and rheological properties as a result of interactions between structural components (calcium and soluble N/total N ratio) were pH dependent (Yazici et al., 2010). Stretching mozzarella cheese curd under high temperature encouraged protein matrix interactions and changes occur in the calcium balance which had an effect on cheese texture (Choi et al., 2008; Gonçalves & Cardarelli, 2021).

Murrah Dairy, a family-run small and medium enterprise (SME), is the pioneer of buffalo dairy production in Thailand. Murrah's dairy farm started in 2003 with 60 heads of Murrah buffaloes with 120 kg/day of raw milk and has since increased to 400 heads in 2022 with 1,600-2,000 kg/day of raw milk. Fresh mozzarella cheese are manufactured at the farm and sold in Bangkok supermarkets and the farm shops. Fresh mozzarella cheese in brine is made daily from whole pasteurized milk. Defects have been observed such as the outer surface of cheese is not smooth during storage in brine and the texture is hard. Therefore, the main objective of this study was to find optimum conditions of milk curd cutting time, cooking temperature of cheese curd and pH of pre-stretched cheese curd to produce a good textural quality mozzarella cheese.

Materials and methods

1. Raw milk collection

Raw milk from Murrah buffalo (*Bubalus bubalis*) was collected and cool-transported from the farm at Plaeng Yao District, Chachoengsao Province, to the dairy processing laboratory at the School of Food Industry, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok. The received milk was sampled for quality analyses. The raw milk was pasteurized at 65ºC for 30 min, cooled and kept in the refrigerator (5 ± 1ºC) until used within 24 - 48 hr.

2. Quality of raw milk

Physical, chemical and microbial properties of raw milk samples were analyzed (American Public Health Association [APHA], 2012). Temperature (°C), clot on boiling test (COB) and specific gravity were determined. The analysis of the fat was conducted by Gerber method (%), protein (%), total solids (TS, %), solids non-fat (SNF), pH and titratable acidity (%TA as lactic acid). Methylene blue reduction test (MBRT), coliform (CFU/mL), standard plate count (CFU/mL), and lactic acid bacteria (LAB) (CFU/mL) were determined.

3. Manufacturing of Mozzarella farm cheese

Commercial condition of Murrah mozzarella farm cheese and literature review was applied. Three batches (replicates) of pasteurized milk of 10 kg each were heated to 40°C. Milk pH was determined before inoculated with lyophilized lactic acid bacteria (*Lactobacillus helveticus* plus *Strepto-coccus thermophiles*) (FD-DVS, Chr. Hansan, Denmark). Milk was thoroughly stirred after inoculation, ripened to a 0.1 pH decrease before rennet enzyme at 0.1 g (Chr. Hansan, Denmark) was added. The temperature was controlled at 40°C until milk curd was developed. The milk curd was cut to approximate 1x1x1 cm and allowed to heat at 40°C for 5 min. The temperature of the cheese curds was gradually increased to cooking at 45°C. The curds were stirred during cooking until pH 6.2 was obtained for whey

drainage, followed by cheddaring and milling, respectively. The milled curds were kneaded and stretched in hot water (> 95ºC), molded into cheese balls of 150 g. The mozzarella cheese balls were stored in 1% NaCl solution at refrigerator temperature and subjected to quality analysis.

4. Determinations of curd cutting time, cooking temperature and pH of pre-stretch curd in mozzarella cheese process

4.1 Determination of milk curd cutting time

Pasteurized milk was inoculated as described above in section 3 and CHY-MAX rennet was added after ripened milk had a 0.1 pH decrease with the temperature controlled at 40ºC. The pH and hardness of milk curd were determined every 15 min during milk ripening. The hardness of milk curd was analyzed by the TA-XT2i Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA) with a cylinder probe (diameter 50 mm). A compression probe attached to a 5 kg compression load cell was used to compress the sample at 1.0 mm/s to a depth of 10 mm from the surface. An optimal curd cutting time was determined by the time of a strong milk curd developed and this cutting time was used in the cheese process for this study.

4.2 Determination of cooking temperature

The cooking temperature was determined by inoculating and renneting pasteurized milk as in Section 4.1, followed by curd cutting, cheese curds and whey were heated and stirred at 3 different cooking temperatures (43, 45, or 47°C) until the pH of whey was about 6.2 (Zisu & Shan, 2005; Gulzar et al., 2019). The optimal cooking temperature was determined by visual assessment of the curds which included adhesion, hardness and stretch ability.

4.3 Determination of pH of pre-stretched curds

After the completion of the cooking step (section 4.2), whey was drained and the cheese curds followed cheddaring. Cheddaring is a process of rebuilding of the cheese mass structure, which is a critical developmental stage of the final taste and texture. The cheddaring steps involved the cutting, stacking and turning of blocks of curd. Cheese curds were agglomerated to curd mass. The curd mass was cut to slabs and stacked on top of each other at 45°C. The stack was turned every 15 min to allow the curd to heat evenly. Whey pH was measured every 10 min until the pH was 5.1 or 4.9 (Gulzar et al., 2019) before milling the cheese mass. The milled curds were kneaded and stretched in hot water (> 95ºC), molded into cheese balls of 150 g. The mozzarella cheese balls were stored in 1% NaCl solution at refrigerator temperature and subjected to quality analysis. Qualities of mozzarella cheese were analyzed, including microbial, chemical and physical quality.

5. Determinations of mozzarella cheese quality

5.1 Microbial quality

Lactic acid bacteria (CFU/mL or g) of the mozzarella cheese were determined (APHA, 2012).

5.2 Chemical quality

Fat content by Gerber (%), protein content (%w/w), solids non-fat (%SNF) and moisture content (%) of mozzarella cheese were determined (APHA, 2012).

5.3 Physical quality

Weight loss (%) of mozzarella cheese was calculated. Textural quality of cheese included hardness, cohesiveness, springiness and chewiness was performed using a TA-XT2i Texture Analyzer. The mozzarella cheese was cut into pieces of 2x2x2 cm. The cheese sample was analyzed by a compression TPA mode, optimized test conditions were 50 mm diameter cylinder; test speed, 1 mm/s; pre-test speed, 1 mm/s; post-test speed, 1 mm/s and distance, 10 mm. Stretch distance (mm) and tension force (N) of the cheese were performed with tension mode by TA-XT2i Texture Analyze with cheese extensibility rig probe.To prepare cheese samples, 5 g of cheese was placed on top of sandwich bread and then baked in an oven at a temperature range of 130-140ºC for a duration of 1.30 min. The melted cheese was measured at 10 mm/s to a height of 120 mm from the surface (modified from Zisu & Shah, 2007).

6. Statistical analysis

Experimental design used in manufacturing of cheese was a completely randomized design (CRD). Results were presented as means ± standard deviation (S.D.) of 3 replications. The data were statistically analyzed using one-way analysis of variance (ANOVA) to determine the curd cutting time, cooking temperature and pre-stretched pH of mozzarella cheese. Means difference was analyzed using Duncan's multiple range tests (DMRT) with a confidence level at 95%. The analysis of the data was performed using SPSS for Windows®, version 16.

Results and discussion

1. Quality of raw milk

Raw buffalo milk was white in color compared to cow milk which was slightly yellow in color. Buffalo milk had a sweet taste and fresh sensation. Physical, chemical and microbiological qualities of buffalo milk are shown in Table 1. Specific gravity (at 20ºC) of Murrah buffalo milk ranged from 1.031 to 1.033 g/cm^3. Qualities of buffalo milk and cow milk in this study were different. Specific gravity of buffalo milk was higher than cow milk (Table 1), this was due to higher fat (6.24%) and SNF (9.51%) contents. The COB test and alcohol test showed negative (-) results indicating the buffalo farm milk was fresh, normal, and stable to heat coagulation. Alcohol test was more sensitive than the COB test. In addition, the alcohol test could be detecting the medium-acidity milk (pH <6.4). Milk which passes the COB test, but it may fail the alcohol test. Colostrum and mastitis milk may also fail the alcohol test (Nurliyania et al., 2015).

The pH (6.68) and titratable acidity (0.15% as lactic acid) of buffalo farm milk was normal. Both pH and titratable acidity of milk depended on feeding, lactation number, stage of lactation, health of an animal and handling of milk at the farm (Şahin et al., 2014; Suranindyah et al., 2015). Buffalo milk had a comparatively higher SNF (9.51%), TS (15.92%), fat content (6.24%) and protein content (3.62%) compared to cow's milk. Milk from Murrah's buffalo had average values of 9.48-10.10%SNF and 15.61-16.53%TS (Meena et al., 2007; Ren et al., 2015; Kapadiya et al., 2016; Sales et al., 2017). Milk fat played an important role in quality of cheese as a plasticizer; a low-fat cheese had a coarse and hard texture compared to a full fat cheese. In addition, milk fat is a good flavor carrier and flavor enhancer. Milk protein was the most important component in buffalo milk manufacturing. Casein, the major milk protein had the lowest solubility at an isoelectric point pH 4.6 (20°C), casein was coagulated while serum protein (whey protein) was soluble. About 80% of the total nitrogen of cow milk and buffalo milk was casein protein. Buffalo milk had a higher casein/protein ratio (80.44%) and high calcium content of casein, this enhanced efficiency of cheese manufacturing (Food and Agriculture Organization of the United Nations [FAO], 2022). Fat and protein contents of raw buffalo milk from Murrah Farm were 6.24% and 3.62% respectively, which compiled to the standard of raw buffalo milk (Thai Agricultural Standard [TAS] 6007-2021). Variations in chemical composition of milk could be due to the species, condition of the animal and the environment (Walstra et al., 2006).

MBRT was performed to indirectly determine the microbial quality of raw milk. The period of the blue color change estimated the number of bacteria in milk. The MBRT of raw buffalo milk took more than 4 hrs and blue color faded in 6 hrs. Results of standard plate count of buffalo milk were 3.4×10^4 CFU/mL which followed the standard (Thai Agricultural Standard [TAS] 6007-2021). Coliform counts of buffalo raw milk were 2.4×10^3 CFU/mL (3.38 log CFU/mL). Microbial qualities of the buffalo farm milk (Table 1) compiled to the standard of raw buffalo milk (Thai Agricultural Standard [TAS] 6007-2021). In some countries in Europe, particularly France, Italy and Switzerland, cheese is manufactured from raw or unpasteurized milk. In Europe the total microbial count in raw milk is required to be less than 5×10^4 CFU/mL (Fox et al., 2017) and the proper management of raw buffalo milk production and handling resulted in good quality of cheese products.

Table 1 Qualities of raw Murrah Farm buffalo milk, standards of raw buffalo milk and cow milk

	Buffalo milk Murrah Farm	Buffalo milk Standard[1]	Cow milk Standard[2]
Physical quality			
Specific gravity (at 20°C) (g/cm^3)	1.032 ± 0.01	≥1.030	≥1.028
Clot on boiling test (COB)	negative	negative	negative
Alcohol test	negative	negative	negative
Chemical quality			
pH	6.68 ± 0.30	6.6-6.9	6.6-6.8
Titratable acidity (%TA)	0.15 ± 0.01	-	≤ 0.16%
solids non-fat (%SNF)	9.51 ± 1.27	≥ 9.0%	≥ 8.25%
Total Solids (%TS)	15.92 ± 2.60	≥ 14%	-
Fat content (%)	6.24 ± 1.08	≥ 5.0%	≥ 3.35%
Protein content (%)	3.62 ± 0.42	≥ 3.5%	≥ 3.0%
Microbiological properties			
Methylene Blue reduction test (MBRT)	> 4 hrs.	> 4 hrs.	> 4 hrs.
Standard Plate Count (CFU/mL)	3.4×10^4	$\leq 4\times10^5$	$\leq 5\times10^5$
Coliform (CFU/mL)	2.4×10^3	$\leq 10^4$	$\leq 10^4$
Thermoduric bacteria (CFU/mL)	$< 10^3$	$\leq 10^3$	$\leq 10^3$

Remark: [1] Thai Agricultural Standard: Raw buffalo milk (TAS 6007-2021)
[2] Thai Agricultural Standard: Raw cow milk (TAS 6003-2010)

2. Effect of cutting time on hardness of milk curd

The relationship of pH and hardness of milk curd based on the time after renneting at 40°C for 75 min is shown in Fig 1. The hardness of the milk curd increased with time, while the pH of the curd decreased

with time which resulted in a stronger milk curd. Efficiency of rennet coagulation and curd formation of cheese milk could be determined by various parameters such as curd tension, curd strength, curd firmness and deformation stress. Several factors influenced milk coagulation by rennet enzymes, such as casein content, fat content, enzyme concentration, pH, calcium ions, size of casein micelles and temperature (Glantz et al., 2010; Fox et al., 2017; Troch et al., 2017).

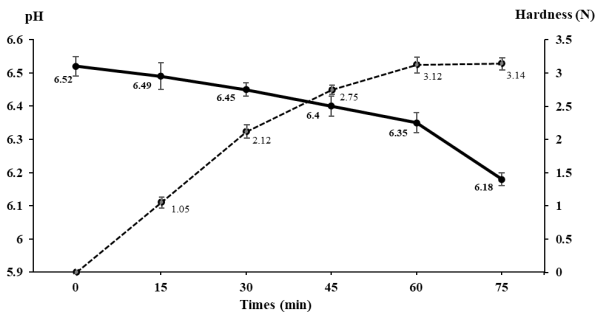

Fig. 1 Relationship of curd hardness (---) and pH (—) of milk after addition of rennet enzyme at 40°C.

Optimal pH range of cheese milk for rennet activity was about 6.0-6.3 (McSweeney et al., 2017) which the rennet activity increased but pH decreased. The optimal temperature for rennet activity was 40-42°C and the enzymatic activity stopped at 55°C (Vignola, 2002; Croguennec et al., 2008). Determination of optimal cutting time by investigating changes in pH and hardness of the milk curd after renneting at 40°C (Fig. 1) showed that a decrease in pH with coagulation time and rennet activity increased the hardness of the curd. Cutting time was important to quality and yield of the final cheese product. At 60 min after renneting pH of the milk curd was greatly reduced from 6.35 to 6.18, with significant increase of the curd hardness to 3.12 N and slowly continued to 3.14 N with constant hardness at 75 min after renneting. Early cutting time (before 60 min) resulted in weak curds with hardness of 1.05-2.75 N. When weak and soft curd was cut before the skin developed, the curds broke up and shattered leading to more fine curds, greater fat loss and cheese yield loss (El-Gawad & Ahmed, 2011). However, longer rennet coagulation time (after 60 min) resulted in poor syneresis of the curds during the cooking stage and increased in cheese moisture (Johnson et al., 2001). Therefore, monitoring pH of curd in addition to coagulation time were applicable guideline for the farm cheese manufacturing. From the result, it is recommended that the curd cutting time for the mozzarella farm cheese at 60 min after renneting, or at pH 6.35 of the milk curd. This result is consistent with the findings of Gulzar et al. (2019) that curd cutting was pH 6.40

3. Effect of cooking temperatures on cheese curd texture

Three levels of cooking temperatures at 43, 45, or 47°C were studied. After milk curd cutting, curds were held at each cooking temperature, while whey was expelled at the same time the curds slowly shrunk. Cooking was controlled until pH of curds was 6.2 before whey drainage. Cooking or heating cheese curd and whey after cutting promoted syneresis, or an expulsion of whey from curd, firming the curd and increased acidity. Cooking temperatures affected curd adhesion, stretch ability and curd hardness (Aldalur et al., 2019). Cutting the curd, curd acidity, cooking temperature and stirring are all factors that promoted the syneresis of cheese curd (McSweeney, 2007). The syneresis was essential to control moisture, to influence texture and flavor of the cheese. Varying the cooking temperature used for production of mozzarella cheese range from 38-44°C. (Yun et al., 1993; AH & Tagalpallewar, 2017).

In this study, cooking curd at 47°C resulted in the hardest curd (Table 2), while at 43°C a soft and non-agglomerated mass curd was obtained. Cooking temperature at 45°C showed suitable hardness of the curds. At 47°C resulted in longer stretched curd with stiff texture (hardness). Therefore, the cooking temperature at 45°C was the optimal temperature. Temperature and pH were mutual contributing factors of curd syneresis. The cooking temperature and cooking rate influenced characteristics of the cheese, increasing the temperature resulted in a dry and hard surface curd due to the moisture loss, decrease in the meltability (Tunick et al., 1993) and the whey was held in the curd, meanwhile low temperature cooking gave a slow rate of syneresis (McSweeney, 2007). Cooking the curd at 45°C until the curd had a pH 6.2, obtained before whey draining and cheddaring, was suggested by Zisu & Shan (2005) and Gulzar et al. (2019).

Table 2 Observation results of cheese curd at various cooking temperatures

	43°C	45°C	47°C
Adhesion	-	+	+
Stretch ability	+	++	+++
Hardness	-	+	++

Remark: - means non detectable
+ means low /++ medium / +++ high detectable

4. Effect of pre-stretched curd pH on quality of mozzarella cheese

Cheddaring rebuilt the curd mass due to restructure of links between casein micelles and fat globules. Texture and structure of curd was developed along with rapid increase of acidity and the built structure was similarly to chicken breast muscle (Mironenko, 2017). In this this study, during cheddaring at 45°C the pH of the cheese curd slowly decreased due to activity of the starter culture. Fox et al. (2017) reported that cheddaring increased ratio of dissolved calcium to calcium bound casein of curd. Soluble calcium (as percentage of total calcium in the curd) was found to increase from 5% to 40% when the pH was decreased from cooking stage to cheddaring stage. In addition, the pH of the curd at cheddaring was the most influential factor in the ability of the curd to be stretched (Yazici et al., 2010; Paz et al., 2017). The pH of the curd, prior to stretching, influenced both textural and microstructural characteristics of the cheese.

Stretching, a process to develop a fibrous texture of mozzarella cheese, included heating, kneading, and curdling cheddared curd. The fibrous structure was not produced if the pH of the curd was higher than 5.8. Buffalo mozzarella cheese was not suitable to stretch if the pH of the curd was higher than 5.2, because fat was lost during the stretching (Gulzar et al., 2019). The pH of the curd, prior to stretching, influenced both textural and microstructural characteristics of the mozzarella cheese.

The quality of mozzarella cheese produced from buffalo milk with pre-stretching at pH 5.1 or 4.9 are shown in Table 3. Different pre-stretched pH of curd did not affect yields or chemical compositions of cheeses. Lactic Acid Bacteria (LAB) counts of raw milk was $<10^3$ CFU/g (data not shown) and LAB of inoculated pasteurized milk was 2.00×10^6 CFU/g and increased to 6.73×10^7 CFU/g during milk ripening or before renneting (data not shown). Chemical compositions of mozzarella cheese obtained from both curd pH 5.1 and 4.9 were similar.

In this study, pH 5.1 or 4.9 of curds at the end of cheddaring (pre-milling or pre-stretching) resulted in different stretching characteristics of the curds and significantly different textural quality of mozzarella cheese (Table 3). At pH 4.9 the stretched curd showed better plasticizing mass during stretching in hot water compared to that of pH 5.1. Curds at pH 5.1 showed the cheese mass had low-stretched curd and the mass of mozzarella cheese was dry and had a rough surface (Fig. 2). However, this defect became smaller in cheese curd at pH 4.9. The results of this study were consistent with the results of the sensory tests of Gulzar et al. (2019), with the sensory score of the cheese appearance at pH 5.1 was less than 4.9, while the higher score preference for texture. Decrease in curd pH was conducive to the flow of the curd during stretching in hot water; this was due to solubilization of micellar calcium phosphate, an increase in the ratio of soluble to colloidal particles of calcium and an increase in para-casein hydration (Guinee et al., 2000; Yazici et al., 2010). Moreover, dicalcium para-caseinate was converted to monocalcium para-caseinate, which favored fiber formation. The curd pH affected the amount of insoluble water calcium which related to unmelted cheese hardness and chewiness of post melts cheese (Yazici et al., 2010; Gonçalves & Cardarelli, 2021).

Textural properties included hardness, cohesiveness, springiness, chewiness and extensibility of mozzarella cheese, was more dependent on pH factor than other factors (Lucey et al., 2003). Even though the cheese curd mass at pH 4.9 showed better plasticizing and easily to form into molding during stretching than pH 5.1. In contrast the texture of mozzarella cheese produced from pH 5.1 showed better quality than pH 4.9. Hardness and chewiness values of the cheese from pre-stretched curd pH 5.1 were lower than that of pH 4.9 (Table 3). Cohesiveness was the maximum extent at which the material can deform before breaking. The cohesiveness values of nearly 1 indicates high cohesiveness or highly rubbery, while nearly 0 indicates no cohesiveness or

(A) (B)

Fig. 2 Outer and inner texture appearance of mozzarella cheese from pre-stretched curd pH 5.1 (A) and pH 4.9 (B)

Table 3 Quality of mozzarella cheese from pre-stretched curds pH 5.1 and 4.9

	pH 5.1	pH 4.9	Other research
Weight loss (%)	13.19 ± 2.52[a]	7.87 ± 1.86[b]	-
Lactic Acid Bacteria (CFU/g)	$<10^4$	3.1 x 10^5	-
Chemical quality			
Fat (%) [ns]	30.38 ± 1.05	29.38 ± 1.49	28.93 (Gulzar et al. (2019); Pignata et al. (2015); Seth & Usha (2015); Andreatta et al. (2009); Sameen et al. (2008))
Fat Dry Basis (%) [ns]	59.91 ± 1.66	57.91 ± 0.80	55.28 (Seth & Usha (2015))
Protein (%) [ns]	18.49 ± 0.44	19.91 ± 1.10	20.56 (Gulzar et al. (2019); Pignata et al. (2015); Seth & Usha (2015); Andreatta et al. (2009); Sameen et al. (2008))
Total Solids (%) [ns]	50.71 ± 2.26	50.73 ± 0.10	53.70 (Gulzar et al. (2019); Pignata et al. (2015); Seth & Usha (2015); Andreatta et al. (2009); Sameen et al. (2008))
Moisture (%) [ns]	49.29 ± 2.26	49.27 ± 0.10	49.11 (Gulzar et al. (2019); Pignata et al. (2015); Seth & Usha (2015); Andreatta et al. (2009); Sameen et al. (2008))
Textural quality			
Hardness (N)	18.03 ± 1.54[b]	21.59 ± 0.34[a]	17.99 (Paz et al. (2017); Pignata et al. (2015); Salama (2015))
Cohesiveness [ns]	0.66 ± 0.05	0.70 ± 0.01	0.50 (Paz et al. (2017); Salama (2015))
Springiness (mm) [ns]	0.75 ± 0.03	0.89 ± 0.15	0.82 (Paz et al. (2017); Pignata et al. (2015); Salama (2015))
Chewiness (N x mm)	9.08 ± 0.46[b]	13.33 ± 2.09[a]	6.10 (Paz et al. (2017); Salama (2015))
Extensibility			
Tension force (N)	0.20 ± 0.01[b]	0.26 ± 0.02[a]	-
Stretch distance (mm)	57.65 ± 4.35[a]	29.45 ± 2.15[b]	46-109 (Hicsasmaz et al. (2004))

Remark: Mean ± SD. with different superscript letters along a row are significantly different ($p<0.05$)
[ns] means not significantly different ($p \geq 0.05$)

highly brittle (Maldonado et al., 2013). In this study, both cheeses showed intermediate cohesiveness. The cheese from pre-stretched curd pH 5.1 showed better extensibility quality of a longer stretchable distance of 57.65 mm, when melted (Table 3), compared to 29.45 mm of pH 4.9 curd. When considering the stretchability of mozzarella cheese after heat baking, it was found that pH influenced the tension force and the stretchable distance. Elongation of mozzarella cheese from pH 4.9 curd requires a higher force than pH 5.1 curd. The stretchable distance of mozzarella cheese mass from pH 5.1 curd was approximately 2 times of the pH 4.9 curd. This corresponded to lower hardness and chewiness quality of cheese from curd pH 5.1 than pH 4.9. The solubility of calcium phosphate in curd had an effect on increasing the hydration of para-casein whose matrix exhibited good fluidity in pH range 6.0-5.2 (Fox et al., 2017). When the pH was closer to the isoelectric point of casein (pH 4.6), para-casein was contracted and decreased hydration that affected the reducing of para-casein matrix flow. Lower casein hydration led the cheese to become hard, rubbery and dry body. (Arora & Khetra, 2017). The ideal pH to plasticize the cheddared curd was 5.15 (Fox et al., 2017). Normally, optimum pH of curd for stretching of pasta filata cheeses type ranged in 5.5 to 5.1 (Rowney et al., 1999; Maldonado et al., 2013; AH & Tagalpallewar, 2017). In addition, the curd pH affected the amount of insoluble water calcium which related to unmelted cheese hardness and chewiness of post melt cheese (Yazici et al., 2010; Gonçalves & Cardarelli, 2021). The stretching step of mozzarella cheese manufacturing gave a unique fibrous texture of pasta filata type cheese. In this study the curd was stretched in the hot water (> 95°C), kneaded until a soft and smooth mass of stretched curd was obtained before molding to 150 g mozzarella cheese balls. Quality comparisons among mozzarella cheese from this study (pre-stretched curds pH 5.1 and 4.9) and those from prior research are shown in Table 3. Chemical compositions of most cheeses were similar. Textural quality of mozzarella cheese from pre-stretched curds pH 5.1 was close to findings of prior research. This supported the study that the optimal pH of the pre-stretched curds was pH 5.1.

Conclusion

Buffalo farm milk had good potential in terms of physicochemical and microbiological characteristics to be used for mozzarella cheese production. Technically, milk curd cutting time, cooking temperature and pre-stretched curd pH were important to qualities of mozzarella cheese. The role of these factors in cheese production cannot be understated, as they are equally important as the knowledge of cheese culture selection. From this study, a 60-min curd cutting time after renneting, 45°C cooking temperature, and pre-stretched curd pH 5.1. were optimal in manufacturing of mozzarella farm cheese from buffalo milk. The success

of this research was applied to quality improvement of mozzarella cheese from Murrah Farm. Finally, this research finding is applicable to SME and industrial cheese production.

Acknowledgments

This research was funded by Thailand Science Research and Innovation (TSRI)and buffalo raw milk was supplied by Murrah Farm, Thailand. The authors are highly grateful for the support by TSRI and Murrah Farm.

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Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Rubber Wood Sawdust Waste Converted to Activated Carbon for Heavy Metal Removal from Wastewater

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Article info

Article history:
Received : 10 January 2023
Revised : 30 March 2023
Accepted : 12 April 2023

Keywords:
Activated carbon, Rubber wood sawdust, Copper ion removal, Adsorption kinetics

Abstract

In this study, we characterized activated carbon prepared from rubber wood sawdust waste and determined optimum conditions for removing copper ions from synthetic wastewater. Rubber wood sawdust was calcined at 600°C for 30 min and then activated with commercial vinegar (5%v/v acetic acid) or 5% lime juice for 24 hr. Three characteristics of the activated carbon were evaluated: (i) to study the ability to remove copper ions versus exposure time, pH and the amount of adsorbent, (ii) adsorption isotherms and (iii) adsorption kinetics. A Langmuir isotherm indicated that the activated carbon adsorbed a monolayer of Cu(II) ions, K_L = 7.91 mg/L with a capacity to adsorb 0.37 mg Cu(II)/g. The optimum conditions for usage was found at 20 g/L activated carbon in wastewater, pH 5 and 60 min adsorption time. Adsorption kinetics were consistent with a pseudo-second order reaction. The results of the study suggest that converting rubber wood sawdust waste to activated carbon allows it to become useful to remove heavy metal pollutants from wastewater.

Introduction

There are many environmental problems that are severe and difficult to solve. One example is sewage, contaminated with heavy metals, leaking into water supplies: water is both a resource and an important factor in the livelihood of all living things and these heavy metals are generally toxic for most life forms. Trace elements can accumulate in many stages of food chains and the ecosystem as a whole, affecting the quality of all living organisms (Renge et al., 2012). Industrial factories uses various heavy metals, including chromium, copper, nickel, zinc and wastewater from these factories contain large amounts of toxic heavy metals (Angthararuk et al.,

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2022). Therefore, factories need to treat their waste effectively and monitor it for pollutants and reduce the toxicity to an acceptable amount before it is released to natural waters. Commercial considerations mean that technology for reducing heavy metal levels in wastewater must be efficient and inexpensive. A method of interest and subject of ongoing research is absorption, as it is inexpensive: an absorption system can be installed easily and quickly, as it uses normal temperature and pressure and no chemical treatment is required (Demey et al., 2018). Activated carbon adsorbents have large surface areas and high porosity and thus high adsorption capacity (Fierro et al., 2008). They can be prepared in various ways, with the main consideration being high carbon and low ash content. A low ash content indicates low concentrations of inactive material: 'activated' carbon implies sufficient surface area and pores, i.e., large absorption area (Banerjee & Mathew, 1985; Wigmans, 1989; Gergova et al., 1994; Pollard et al., 1995; Namasivayan & Kadirvelu, 1997; Kadirvelu et al., 2000). It can be formed from several natural materials – ranging from stones and seeds to husks, shells and bones (Thomas & George, 2015; Zazouli et al., 2016).

Thailand is an agricultural country and as such agricultural wastes are plentiful and scattered throughout the country, with an estimated 43 million tons per year of non-used agricultural waste (Tengkaew & Wiwattanadate, 2014). Agricultural waste can be used to produce as activated carbon, since its main constituent is carbon. It is readily available locally, cheap and environmentally friendly (Ioannidou & Zabaniotou, 2007; Saygili et al., 2015). Examples include oil palm shells (Hesas et al., 2013), rice husk (Saygili et al., 2015), sugarcane bagasse (Gurgel et al., 2008; Junior et al., 2009), coconut husks and corncobs (Tan et al., 2008). Other methods to remove heavy metals include chemical precipitation, ion exchange, chemical oxidation, reduction, reverse osmosis, ultrafiltration and electrodialysis (Fu & Wang, 2011). Recently, Muzarpar et al. (2020) and Wang et al. (2023) reviewed adsorption by activated carbon from natural sources.

Rubber plants are important crops in the Thai economy: farmers and rubber-related businesses involve at least 1 million and possibly as many as 6 million families if all related businesses are counted. Since 1991, Thailand has been the world's leading exporter of rubber and rubber products. Thailand currently faces the problem of low prices for automobile tires, a major user of natural rubber. Thus, the income of rubber farmers has been affected and the government has begun to promote the rubber wood industry. The promotion of rubber tree exports is due to the situation that in many countries the forests have been closed to cutting trees, causing a shortage of timber. As a result, rubber trees are in greater demand from Thailand. In addition to exporting rubber wood, it is still processed in the furniture industry and has experienced an increase in demand every year (Sangsuwan et al., 2019). Rubber trees that are tapped to generate natural rubber lose their efficiency with age and traditionally trees have been burnt after about 25 years. Currently the rubber trees provide further supply for the furniture industry which has caused a large amount of waste in the form of sawdust.

Therefore, the aim of this research was to show a further economic use for rubber tree sawdust such as being turned into activated carbon and applied to adsorb copper (and similar heavy metals) in wastewater. The research also focused on an economic technique to enhance pollutant adsorption using readily available commercial products. The study focused on an alternative way to add value to local agricultural waste in order to maximize economic benefits as well as environmental benefits.

Materials and methods

1. Materials

The raw materials and equipment used in this research are detailed in Table 1 and Table 2.

Table 1 The sources of raw materials

Material	Source	Note
Rubber wood sawdust	Mill, Kalasin, Thailand	16.43986°N, 103.50657°E
$Cu(NO_3)_2.3H_2O$	Carlo Erba	AR grade
NaOH	Carlo Erba	AR grade
HCl	Carlo Erba	AR grade
Acetic acid or vinegar	Commercial vinegar 5%v/v acetic acid	Local store
Lime	Commercial lime 5%v/v lime juice	Local store
Activated carbon	Chemipan Corporation Ltd.	Commercial activated carbon

Table 2 The equipment used

Item	Manufacturer
Oven	Memmert UFB400, Germany
Furnace	Carbolite RHF1600, Germany
UV-VIS spectrophotometer	Perkin Elmer Lambda 12, Germany
Scanning Electron Microscope	JEOL JSM-7800F, Germany
FTIR Spectrometer	Perkin Elmer Spectrum GX, Germany
Hotplate & Stirrer	Jenway 1103, United Kingdom

2. Preparation of activated carbon

Crushed dried rubber wood sawdust at 50 g was carbonized at 600°C for 30 min and activated with 500 mL commercial lime or acetic acid for 24 hr. The charcoal was filtered out and washed several times with distilled water until pH of the washing water was ~7. The charcoal was dried for 2 hr and incinerated (600°C, 60 min). After that, iodine adsorption analysis determined the activated carbon produced (Test D 4607, ASTM, 1998a). The prepared products were subjected to proximate analyses (ASTM, 1998b; ASTM, 1998c), morphology was examined by a scanning electron microscopy. Activated carbon was prepared at sintering temperatures from 300 to 700°C with calcination times from 30 to 180 min.

3. Wastewater preparation and standard calibration curve with Cu(II) solution

A synthetic wastewater was prepared from $Cu(NO_3)_2.3H_2O$-a pollutant common in industrial effluent, by dissolving 0.12 g $Cu(NO_3)_2.3H_2O$ in distilled water in a 1 L measuring flask to form a 0.5 mM solution. Cu(II) reference solutions were prepared at concentrations of 30, 40, 50, 60, 70, 80, 90, 100,110 and 120 ppm of $Cu(NO_3)_2.3H_2O$, by dilution from an initial 120 ppm Cu(II) solution. Using a UV-VIS spectrophotometer, absorbance of these solutions, at 560 nm, using a distilled water reference, formed a linear calibration curve, $R^2 = 0.9921$.

4. Adsorption studies

We assessed the effects of exposure time, pH and adsorbent loading. FT-IR spectra of the activated carbon showed relatively higher fractions of oxygenated groups (strong bands at 3340 and 1020 cm^{-1}) compared to commercial activated carbons. This result could possibly be attributed to stronger heating and drying to drive out volatile organics in the commercial products, which also showed considerably higher fixed carbon content (see Table 3).

4.1 Effect of time

To determine the optimal contact time for adsorption of Cu(II), we used the synthetic water, 0.5 mM Cu^{2+} or ~30 µg Cu/L, pH 7, an activated carbon loading of 5 g/L and exposure times of 15, 30, 45, 60, 75 and 90 min.

4.2 Effect of pH

For optimum pH determination, we used the same synthetic water and activated carbon loading and the optimum exposure time (60 min from item 4.1). The pH was adjusted with hydrochloric acid and sodium hydroxide: values were set at 3, 5, 7, 9 and 12.

4.3 Effect of adsorbent loading

For adsorbent loadings, we used the same procedure and optimum conditions, found in sections 4.1 and 4.2, with activated carbon loadings of 0.5, 1, 1.5, 2, 2.5 and 3 g. We calculated the adsorption and the adsorption capacity as follows:

$$\%\,\text{Adsorption} = \left(\frac{C_o - C_e}{C_o}\right) \times 100 \quad (1)$$

$$q_e = \left(\frac{C_o - C_e}{W}\right) \times V \quad (2)$$

where C_o = initial solute concentration (mg/L), C_e = residual concentration of the solute (mg/L), q_e = amount of pollutant absorbed per gram of adsorbent (mg/g), V = solution volume (L), w = adsorbent mass (g).

5. Isotherm models

The study of copper ion adsorption kinetics with activated carbon prepared from rubber wood sawdust using concentrations of Cu(II) solutions from 60 to 100 mg/L. The activated carbon content was 0.5 g, the pH value was 5 and the exposure time was 60 min. The results were recorded on a plot graph showing the Langmuir and Freundlich adsorption isotherms.

5.1 Langmuir model

The Langmuir isotherm for monolayer adsorption assumes that only one molecule can be adsorbed on each adsorption site, each position has the same heat of adsorption and no forces act between neighbouring molecules. A model for this behaviour can be formally described in Eq. 3 which defines the amount of the adsorbate on the solid surface, q_e (Sarvestani et al., 2016; Mousavi et al., 2018; Foroutan et al., 2019) using:

$$\frac{1}{q_e} = \frac{1}{bK_L C_e} + \frac{1}{K_L} \quad (3)$$

where K_L = highest monolayer capability, C_e = metal concentration and *b* is a constant.

A plot of $\frac{1}{q_e}$ vs $\frac{1}{C_e}$ will lead to a straight line with intercept $\frac{1}{K_L}$, and slope, $\frac{1}{bK_L}$.

5.2 Freundlich model

An entirely empirical model, the Freundlich model is used for adsorption on rough surfaces, where each surface may produce varying heats of adsorption. A Freundlich isotherm assumes that q_e is proportional to a concentration raised to a 1/n power (Foroutan et al., 2019):

$$q_e = kC_e^{1/n} \quad (4)$$

where k and n are empirical constants, with q_e and C_e defined in Eq. 3. Taking logarithms of Eq. 4, the Freundlich model leads to:

$$\log q_e = \log k_F + \frac{1}{n}\log C_e \quad (5)$$

so that plotting log (q_e) vs log (C_e) at constant temperature leads to a line from which k can be derived from the y-intercept and n from the slope, which is 1/n (Foroutan et al., 2019).

6. Kinetic studies

To study copper adsorption kinetics with the activated carbon prepared from rubber wood sawdust, we used a 0.414 mM Cu (II) solution 10 mg Cu $(NO_3)_2.3H_2O$/L. We added 1.0 g activated carbon to a 50 mL volume, pH 5 and sampled every 15 min for 90 min. The plots for pseudo-first and pseudo-second reactions are shown in Eq. 6 and Eq. 7 (Sarvestani et al., 2016; Mousavi et al., 2018).

6.1 Pseudo-first order kinetic model

For first order kinetics, we expect:

$$\log (q_e - q_t) = \log q_e - k_1 t \quad (6)$$

where q_e and q_t are the amounts of copper ion adsorbed (mg/g) at equilibrium, t is time (min) and k_1 is the pseudofirstorder rate constant. The values of q_e and k_1, governing the sorption of copper ion from aqueous solution onto adsorbent, were determined from the intercept and slope of the plot of $\log(q_e - q_t)$ *vs* t.

6.2 Pseudo-second order kinetic model

Second order kinetics is described by:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad (7)$$

where, k_2 is the pseudo-second-order rate constant, determined from a plot of t/q_t vs t.

Results and discussion

1. Activated carbon preparation

The preparation of 50 g charcoal, calcined at 600°C for 30 min, was ground thoroughly and soaked with 500 mL of commercial line or vinegar for 24 hr, after which the charcoal was filtered and washed with distilled water until the wash water pH reached 7. Then 5 g charcoal was dried for 2 hr and incinerated at 600°C for 60 min. After that, the iodine adsorption of the activated carbon was analyzed-see (Fig. 1)

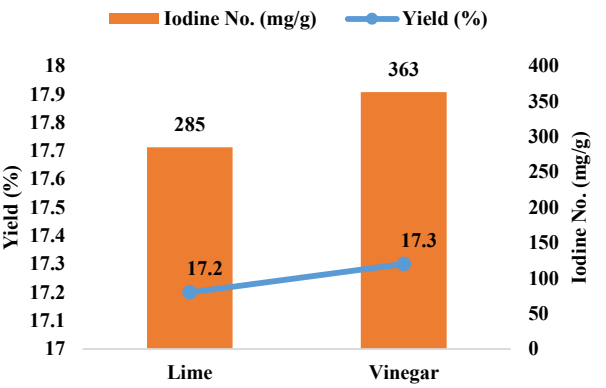


Fig. 1 Yield and iodine adsorption of activated carbon

Fig. 1 compares lime and acetic acid activation: activated charcoal with lime had an iodine adsorption value of 363 mg/g and with vinegar it was 285 mg/g. Acetic acid led to activated charcoal that was more porous than that activated by lime water. Therefore, vinegar was used to activate the carbon from rubber wood sawdust in the next process.

Fig. 2 shows iodine adsorption after activation with 5%v/v acetic acid vs calcination temperatures from 300 to 700°C for 60 min. Iodine adsorption increased with temperature leading to a maximum 366 mg/g at 600°C. A slight decrease was observed at 700°C, 344 mg/g. We attributed this to temperatures above 600°C increasing the pore size and concomitantly decreasing the active surface area. Therefore, 600°C was chosen to prepare the activated carbon for determination of the appropriate time.

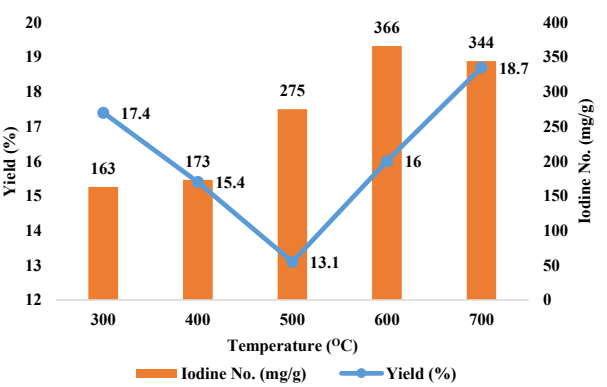


Fig. 2 Iodine adsorption and yield *vs* calcined temperature

From Fig. 3, after calcining at 600°C for up to180 min, it was found that the activated carbon prepared by incineration at 60 min had the highest iodine adsorption value, 366 mg/g. If the time was longer the iodine adsorption reduced. This was attributed to pores coalescing, so that the number and total area of pores decreased, resulting in a decrease in yield and iodine adsorption. Therefore, 60 min was chosen as the calcination time to prepare activated carbon for use in the copper ion solution adsorption experiments.

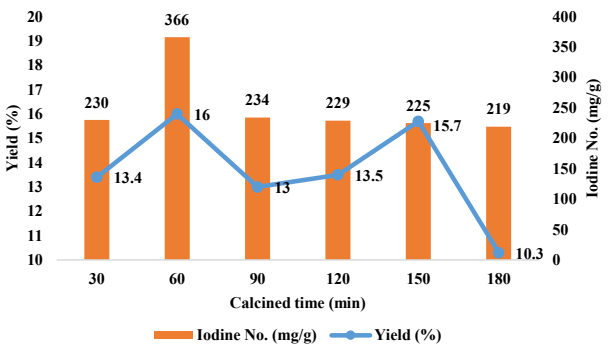


Fig. 3 Iodine adsorption and yield *vs* calcined time

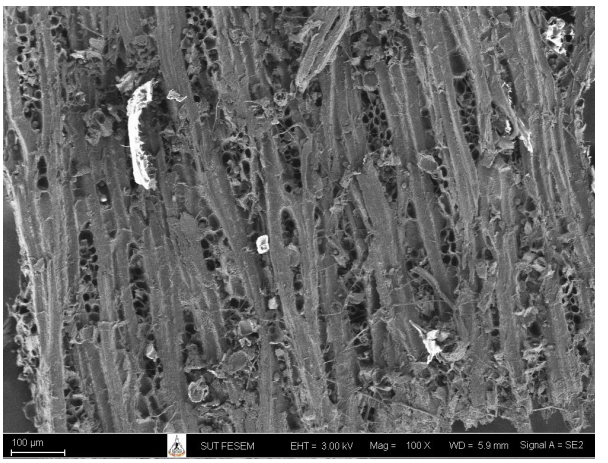
2. Properties of activated carbon from rubber wood sawdust

Table 3 shows that untreated rubber wood sawdust had the highest volatile matter content, followed by sawdust calcined (600°C, 30 min) and sawdust treated with acetic acid and re-calcined (600°C, 60 min), indicating that most of the readily degradable organics were volatile at high temperatures. This was attributed to decomposition of hemicellulose and cellulose components (Guo & Lua, 2001), whereas the ash content was inversely related to the stable carbon content. The prepared activated carbon appeared as a black powder.

Scanning electron micrographs (100 × and 1000 × magnifications) (Fig. 4) revealed the pore structure of the sawdust and activated carbon.

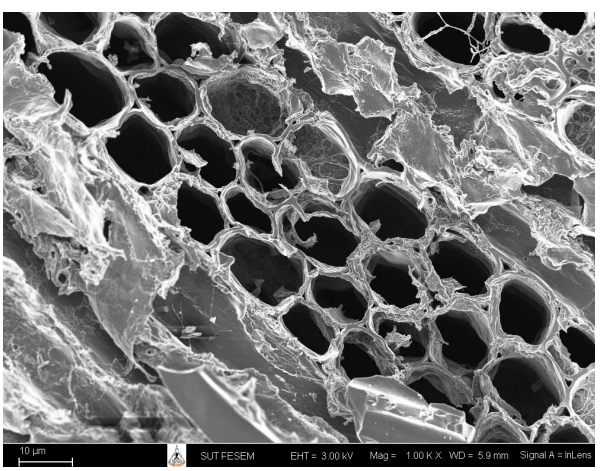
Table 3 Approximate composition analysis.

Type	% moisture	Approximate composition (% by weight)		
		Ash	Volatile	Fixed carbon
Untreated rubber wood sawdust	10.8	36.0	42.0	11.2
Sawdust, calcined at 600°C for 30 min	3.5	26.5	24.0	46.0
Sawdust soaked in acetic acid, 24 hr, calcined, 600°C for 60 min	2.1	24.6	20.6	52.9
Commercial activated carbon	1.0	16.4	1.4	81.2



(a) Untreated sawdust,100 × mag

(b) Sawdust, calcined 600°C, 30 min, 1,000 × mag



(c) Sawdust, soaked in acetic acid 24 hr, calcined 600°C, 60 min, 1,000 × mag

Fig. 4 Surfaces: raw materials and activated carbon

The surface characteristics of untreated rubber wood sawdust (Fig. 4a) differed from treated sawdust (Fig. 4b and 4c). The untreated rubber wood sawdust had a relatively flat surface and nested in layers (Fig. 4b and 4c). Sawdust, calcined at 600°C for 30 min and sawdust, soaked in vinegar for 24 hr calcined at 600°C for 60 min and had porous surfaces. The sawdust that had been calcined and soaked in vinegar had a more porous surface, attributed to acetic acid's ability to break down lignin and also destroy the cellulose crystalline structure, resulting in a more textured and porous sawdust. The increased number of pores correlated with the sorption ability. This depends on matching the pore size and the size of the adsorbent. If the adsorbent pores are smaller than the pollutant molecules sorption will be low (Al-Anber, 2010; Osu & Odoemelam, 2010). For example, Duy Nguyen et al. (2019) prepared activated carbon from sawdust for adsorption of Cu and Cd from solution. The activated carbon adsorbed Cu more strongly than Cd. Ion exchange depends partly on the atomic radius: the Cd ion radius is 9.7 nm, or larger than that of Cu, 7.3 nm. This implies that our results were consistent with the relative ionic radii (Duy Nguyen et al., 2019).

3. Infrared spectra

FTIR spectra are shown in Fig. 5. Whereas Fig. 5a shows the untreated sawdust had broad stretching peaks at 3340 cm^{-1} (O-H), 2913 cm^{-1} (C-H) and 1030 cm^{-1} (C-O in alcohol, phenol and aliphatic ether groups). Stretching for unconjugated C=O groups were seen at 1727 cm^{-1} and a strong peak at 1595 cm^{-1} was assigned to C=O attached to an aromatic ring. A peak at 1325 cm^{-1} arose from bending of the syringly group, a basic lignin component. After calcining at 600°C, OH peaks reduced significantly, the CO peak at 1030 cm^{-1} almost completely disappeared, confirming loss of alcohols, phenols and water, i.e., components which are volatile or decompose on heating. However, the C=O attached to an aromatic ring remained (peaks at 1715 cm^{-1} and 1588 cm^{-1}).

Activation with acetic acid before calcining removed some residual aliphatics (band at 1384 cm^{-1} now absent), but absorption at 1717 cm^{-1} and 1592 cm^{-1} indicated that some aromatic components remained. The commercial activated carbon exhibited small amounts of aromatics, similarly to both calcined samples but alcohols and phenols were almost absent.

The commercial activated carbon (Fig. 5d) showed peaks at 1595, 1632 and 1669 cm^{-1} assigned to carbon (graphite) and no hydroxy peaks at 3320 cm^{-1} or C-O bending peaks at 1030 cm^{-1}. Acetic acid activation resulted in a structure that closely resembled commercial activated carbon, as opposed to unactivated sawdust. Additionally, the efficacy of the acetic acid-activated product was comparable to that of commercial activated carbon, suggesting that it could potentially serve as a viable substitute for the latter.

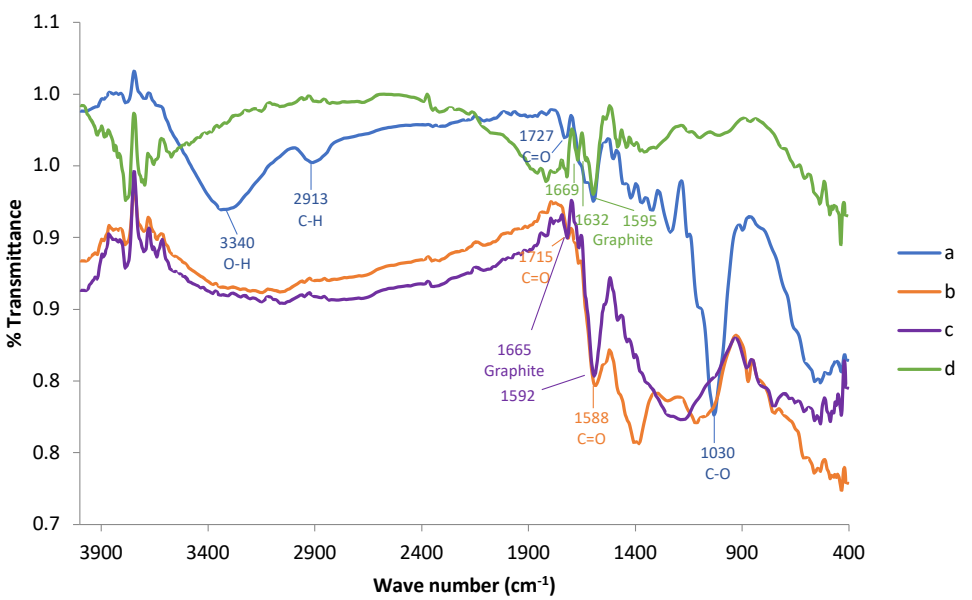


Fig. 5 FT infrared spectra of activated carbons prepared in this study and a commercial activated carbon (a) Untreated rubber wood sawdust (b) Rubber wood sawdust, calcined 600°C for 30 min (c) Rubber wood sawdust, soaked in acetic acid for 24 hr, calcined at 600°C, 60 min (d) Commercial activated carbon

4. Adsorption capacity

The adsorption capacity of the activated carbon produced from rubber wood sawdust was evaluated under various conditions namely, the activated carbon loading, pH and time and the adsorption isotherm was determined and compared with commercial activated carbon. To study the influence of time on adsorption, the initial concentration of Cu(II) was 0.412 mM, 0.5 g of activated carbon was loaded in100 mL, pH was 7.0 and it was shaken with an automatic shaker for 15 to 90 min.

From Fig. 6, adsorption increased rapidly with contact time to 60 min: the copper adsorption rose from an initial 7.5 to 42.5 at 60 min. At this point, it reached equilibrium and we observed a slightly reduced fraction of adsorption. The higher the contact time, the higher the adsorption value (Reza et al., 2014). This is due to the increased rate of diffusion on the surface of the adsorbent and enters equilibrium when the adsorption rate is constant (Ouyang et al., 2019).

Fig. 7 shows the effect of pH on adsorption: synthetic water, loaded with 5 g/L of adsorbent from pH 3 to 12 were adjusted with acid or base and shaken for 60 min. %adsorption *vs* pH is shown in Fig. 7.

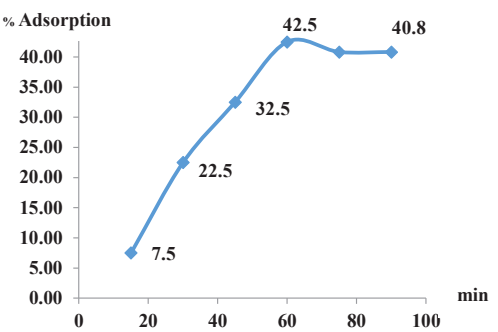


Fig. 6 Influence of Adsorption time

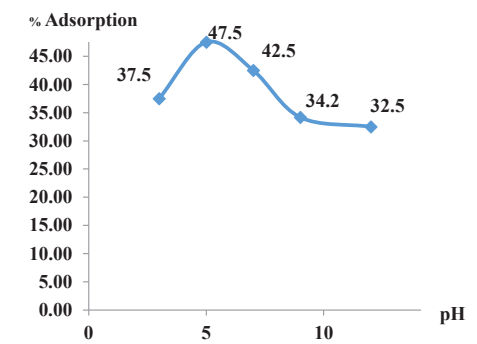


Fig. 7 Influence of pH

As the pH increased from 3 to 5, the copper adsorption capacity increased from 37.5% to 47.5%, but decreased for pH > 5, attributed to formation of negative hydroxyl complexes of copper at higher pH (Chen et al., 1996; Chen & Lin, 2001; Noh & Schawars, 1990).

$$[surface]\ xH_2O + M^{n+}(aq) \leftrightarrow [M(H_2O)x]^{n+} \leftrightarrow [surface]\ [M(H_2O)_{x-1}OH]^{(n-1)+} + H^+(aq)$$

These copper complexes are now larger and less able to enter pores on the activated carbon surface, reducing adsorption (Duan et al., 2016; Ajmal et al., 2003). Also, as shown by our IR spectra, the residual compounds in activated carbons vary widely with different raw materials and conditions, thus the optimum pH for copper adsorption may vary with the carbon source due to their abilities to repel Cu complexes (Al-Senani et al., 2018; Banat et al., 2002; Lorena et al., 2020; Onundi et al., 2010; Monser & Adhoum, 2002). In the Cu(II) adsorption experiment, 100 mL of the synthetic water at pH 5.0 was shaken with an automatic shaker for 60 min with activated carbon loadings varying from 0.5 to 3 g (i.e., 5 to 30 g/L).

As Fig. 8 shows, when activated carbon loading increased from 5 g to 20 g/L, adsorption increased from 47.5% to 62.5%, due to the increased activated carbon surface. However, above 20 g/L, additional adsorbent showed no benefit and may have prevented complete interaction with the solution.

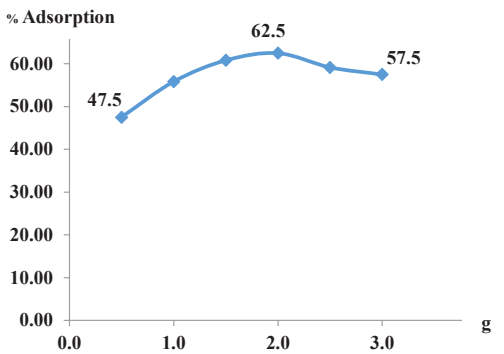


Fig. 8 Influence of adsorption loading (g activated carbon/100 mL)

5. Adsorption isotherms

To elucidate the underlying adsorption mechanism, Langmuir and Freundlich isotherms were compared - see Eq. 4 and Eq. 5. Copper solutions at concentrations of 60, 70, 80, 90 and 100 mg/L, activated carbon loading 5 g/L, pH 5, were shaken for 60 min. After activated carbon

was filtered out, the remaining copper concentration was measured in the supernatant. Data was transformed to $1/q_e$ and $1/C_e$ for the Langmuir model (Fig. 9) and log (q_e) and log (C_e) for the Freundlich model (Fig. 10).

For the Langmuir model in Fig. 9, the regression line had a slope, $1/bK_L$ = 0.3392 and a y-intercept, $1/K_L$ = 0.1264, K_L = 7.91, indicating the adsorption capacity maximum with a monolayer surface. Adsorbent ions or molecules could not cross the surface, nor bind to adjacent molecules (Taty-Costodes et al., 2003). The Freundlich model produced a regression with slope, 1/n = 2.5586 and y-intercept, log K = 0.39. n < 1 indicated that the amount of surface adsorption was limited (Ajemba, 2014).

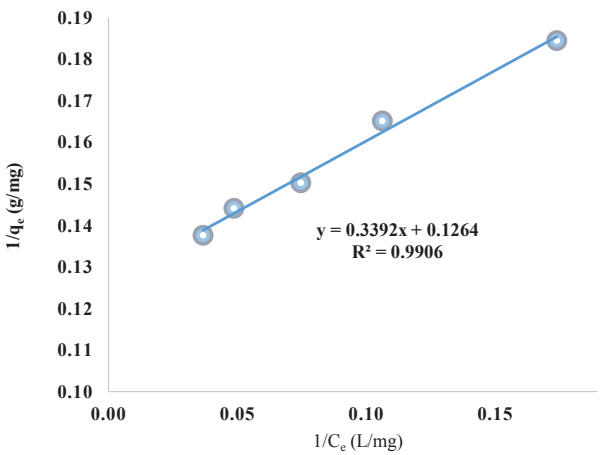


Fig. 9 Langmuir copper adsorption isotherm

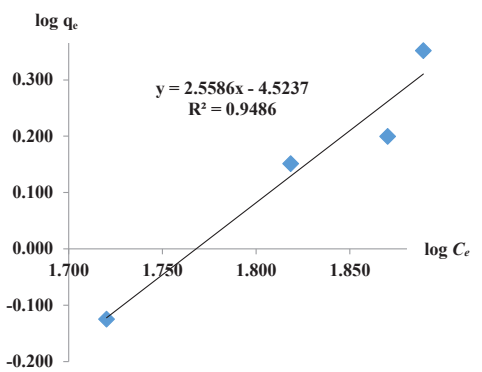


Fig. 10 Freundlich adsorption isotherm

The Langmuir adsorption isotherm, with R^2 = 0.99, was clearly a better representative of the mechanism, i.e. a monolayer of Cu^{2+} ions was formed on the surface and showed a 77% surface coverage.

Activated carbon from a wide variety of natural sources has been studied extensively, Table 4 compares critical parameters from our rubber wood derived activated carbon compared with other lignite-heavy (woody) sources. Most sources exhibited Langmuir model absorptions, but Kadirvelu et al. (2000) and Uzun & Guzel (2000) reported Freundlich adsorptions from 'commercial' sources. Although, the measured adsorption capacity is low, the process we used was extremely simple and used weak, commercially available acids, compared to the stronger and more expensive, ones, typically H_3PO_4, used by others, this lead to smaller pores restricting Copper ion adsorption.

Table 4 Adsorption capacities from woody biomass sources

Study	Source	Adsorption capacity (mg/g)	Langmuir K_L (L/mg)
Kadirvelu (2000)	Commercial 'cloth'	1.1 - 8.5	0.73 - 1.08
Rafutullah (2009)	Meranti sawdust	32.1	0.05
Demiral & Ayan (2011)	Grape bagasse	37.2 - 43.5	0.11 - 0.28
Duy Nguyen (2019)	Teak wood	159 - 182	0.031 - 0.062
Lee (2019)	Gingko leaf	59.9	
Patel (2020)	Neem leaf	154	0.064
This study (2023)	Rubber wood	0.37	7.91

6. Adsorption kinetics

For assessing the adsorption kinetics, prepared 50 mL synthetic water, loaded with activated carbon 10 g/L, pH at 5, was sampled every 15 min until 90 min. Remaining Cu in the solution was measured. Data was transformed to match a first order model (Eq. 6) (Kaczala et al., 2009) and plotted in Fig. 11. Similarly, consistency with a second order model (Eq. 7) (Vadivelan & Kumar, 2005) as shown in Fig. 12.

A second order model was a better fit (R^2 > 0.9957) than the first order model (R^2 = 0.7982). This indicated that copper adsorption by activated carbon prepared from rubber wood sawdust was chemical adsorption, i.e., chemical bonds or chemical forces occurred by exchanging electrons between metal ions and the adsorbent (Semerjian, 2018).

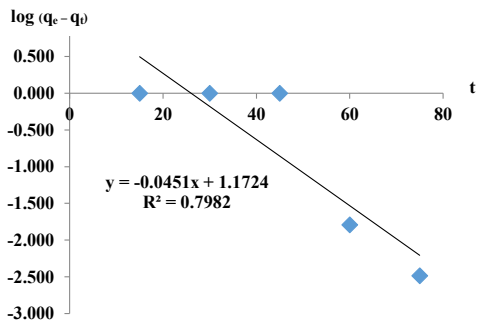


Fig. 11 Pseudo-first order model: log (q_e-q_t) *vs* t

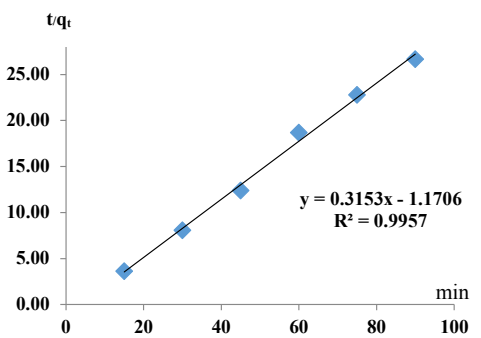


Fig. 12 Pseudo-second order model: t/qt *vs* t

Conclusion

Optimal conditions for a simple and inexpensive process to produce activated carbon from rubber wood sawdust, where commercially available vinegar (acetic acid) showed a better activator than a lime solution. The optimum sintering needed calcining at 600°C for 60 min with acetic acid as an activator. Electron microscopy showed that this activation led to a more porous surface, because acetic acid was able to break down lignins in the raw material, leading to a 27% greater surface area, measured by iodine adsorption. The generated activated carbon was able to remove more than 40% of the Cu ions in synthetic wastewater at 5 g activated carbon per L, with removal increasing to a peak of 60% on higher loadings (20 g activated carbon/L). Comparison between Langmuir and Freundlich adsorption models supported a mono-layer adsorption pattern, i.e., a Langmuir model, with K_L = 7.91 L/g, adsorbing 0.37 g Cu(II) per g of activated carbon. Adsorption kinetics showed that a pseudo-second order model fitted the data better, hence our conclusion of per g of activated carbon that chemical bonding between Cu(II) ions and the activated carbon surface dominated.

A readily available waste such as sawdust from rubber wood furniture manufacture was converted to an effective adsorbent for metal ions in wastewater. A pre-treatment with acetic acid, from commercial vinegar, was more effective in increasing the number of pores and thus the adsorption of the activated carbon. The raw material has a negative market value, because disposing of it requires costs and burning it will create unneeded pollution, whereas converting it to activated carbon allows it to become useful to remove heavy metal pollutants from wastewater. Pretreatment requires only natural chemicals. The adsorption kinetic parameters may be used to guide larger scale commercial treatment plants.

Acknowledgements

The authors acknowledge Research and Development Institute, Rajabhat Maha Sarakham University for financial support and thank the Center for Scientific and Technological Equipment, Suranaree University of Technology for acquiring the FT-IR and SEM data.

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Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Lipid Production of Marine Green Microalgae *Chlorella protothecoides* BUUC1601 by Using Spent Coffee Grounds Hydrolysate

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Article info

Article history:
Received : 5 March 2023
Revised : 18 April 2023
Accepted : 23 April 2023

Keywords:
Fatty acid, Marine green microalgae, *Chlorella protothecoides*, Lipid production, Spent coffee grounds hydrolysate

Abstract

Spent coffee grounds are an organic waste that can be used as a source of microbial organic carbon. In this research, coffee grounds were hydrolyzed into a solution called spent coffee grounds hydrolysate (SCGH) using concentrated sulfuric acid. Then, the marine green microalgae, *C. protothecoides* BUUC1601, was cultured using SCGH. Growth performance and lipid accumulation of the microalgae were evaluated. The microalgae were cultured using a standard F/2 medium without and with SCGH added in the range of 2.5-15% of culture media (v/v). It was found that the microalgae had similar growth performance and biomass yield, i.e., the specific growth rate was in the range of 0.87-1.12 day^{-1} and the biomass yield was in the range of 0.05-0.08 g DW/L/day. Microalgae cultivation using F/2 with SCGH had an effect on lipid accumulation. It was found that using SCGH at 15% of the total volume resulted in an increase in the amount of lipid accumulation up to 66.03% of the dry weight. Of these, it was 2.89 times higher than the lipid content of microalgae cultured with no SCGH added. The content of monounsaturated fatty acids ranged from 46.15% to 46.53% and polyunsaturated fatty acids ranged from 32.40% to 34.62% of the total fatty acid content. Oleic acid (C18:1n9), an omega-9 fatty acid, was found to be the most abundant, accounting for over 30% of the total fatty acid content. In contrast, the omega-6 fatty acids linoleic acid (C18:2n6) and gamma-linolenic acid (C18:3n6) were found to be lower, accounting for 25.99%-27.20% and 6.03%-7.01% of the total fatty acid content, respectively. The omega-3 fatty acid such as alpha-linolenic acid (C18:3n3)

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was found at 4.20% in microalgae cultured using standard F/2 medium without SCGH, which was higher than in microalgae cultured with the addition of SCGH. Therefore, the addition of SCGH at a concentration of 2.5%-15% (v/v) can be used to cultivate *C. protothecoides* BUUC1601 for the lipid production with high unsaturated fatty acid content, which has the potential to be used in both aquaculture and functional food supplementation.

Introduction

Coffee drinks are popular all over the world and coffee consumption is forecasted to increase. Coffee consumption is in the form of instant coffee from the industrial sector, and fresh coffee from both households and coffee shops. Both types of coffee, instant coffee and fresh coffee, require roasted coffee beans, grinding and extraction with hot water (Cruz-Lopes et al., 2017). The production of instant coffee and fresh coffee therefore has a waste product in the form of spent coffee grounds (SCG), which increases with the demand of coffee consumption. However, coffee grounds are organic compounds, if not disposed of properly may be contaminated and have a negative impact on the environment (Ballesteros et al., 2014). Coffee grounds contain lignocellulose which is the main component such as cellulose, hemicellulose and lignin at 10.78%, 28.36% and 10.72% by weight, respectively. In addition, SCG contain protein, ash and tannin at 9.28%, 1.8% and 30.36% by weight, respectively. Thus, coffee grounds still remain as useful biological compounds especially in the form of insoluble compounds such as sugars that are composed of cellulose and hemicellulose i.e. glucose, galactose, mannose and arabinose (Cruz-Lopes et al., 2017; Scully et al., 2016). SCG can be used in the form of solid coffee grounds directly such as fertilizer because of the good water holding capacity and rich in organic matter and trace elements. Moreover, SCG also can be used as spent coffee grounds hydrolysate (SCGH). Which is a liquid form of the digestion of coffee grounds (hydrolysis) by using chemicals such as sulfuric acid or enzymes i.e. mannanase, endoglucanase, exoglucanase, xylanase and pectinase. Therefore, SCGH is rich in sugars that can be used in related fields such as biofuel production, amino acid and enzyme production and microorganism's biomass production as well arabinose (Scully et al., 2016; Jooste et al., 2013; Hudeckova et al., 2018).

Chlorella protothecoides is the marine green microalgae that has a high nutritional value and is able to grow in autotrophic, mixotrophic and heterotrophic conditions. In autotrophic growth conditions, microalgae can produce their own food through photosynthesis by using carbon dioxide gas as a carbon source. While in mixotrophic and heterotrophic growth conditions, microalgae use carbon sources in the form of organic substances such as sugars, fatty acids and acetate. For mixotrophic growth, microalgae required light during the culture period in contrast to the heterotrophic growth where microalgae can grow without light (Heredia-Arroyo et al., 2010). It has been reported that cultured *C. protothecoides* in mixotrophic and heterotrophic growth conditions can have an effect on lipid accumulation better than cultured under autotrophic growth conditions. Therefore, unsaturated fatty acids in *C. protothecoides* will increase such as oleic acid (C18:1n9), linoleic acid (C18:2n6) and gamma-linolenic acid (C18:3n6). These polyunsaturated fatty acids are very important in biofuel, nutraceutical and pharmaceutical (Da Rosa et al., 2020).

In this research, SCG was collected from coffee shops in Mueang District, Chanthaburi Province, Thailand. The SCG was hydrolyzed with concentrated acid to prepare SCGH for lipid production of *C. protothecoides* BUUC1601. Microalgae can be used in related fields such as aquaculture, functional food and biofuel production. In addition, the result of this experiment provides suggestions for usage of SCG which can be created from the waste from coffee shops, domestic and coffee industry to be more functional.

Materials and methods

1. Microalgae strain

Marine green microalgae *C. protothecoides* BUUC1601 in this experiment was isolated from the coastal area in Chanthaburi Province (Phirulpawadee, 2016). The microalgae inoculum was maintained at the Marine Biotechnology Research Unit, Faculty of Marine Technology, Burapha University. It was grown in standard F/2 medium (Guillard, 1973) with a salinity at

30 psu and under constant light using cool-white, fluorescent lamps at 80 µM/m²/s.

2. Culture medium

Standard F/2 medium (Guillard, 1973) was used for this research. The medium consisted of the following components (per liter of seawater): 75 mg $NaNO_3$, 5 mg $Na_2HPO_4.H_2O$, 30 mg $NaSiO_3.9H_2O$, 0.436 mg Na_2 EDTA, 0.315 mg $FeCl_3.6\ H_2O$, 0.01 mg $CuSO_4.5H_2O$, 0.022 mg, ZnSO4.7H_2O, 0.01 mg $CoCl_2.6H_2O$, 0.18 mg $MnCl_2.4H_2O$, 0.006 mg $Na_2MoO_4.2H_2O$, 0.2 mg Thiamine-HCl, 1 mg Biotin and 1 mg Cyanocobalamin.

3. SCG source

Arabica coffee grounds were obtained by courtesy of a coffee shop in Muang District, Chanthaburi Province, Thailand and used throughout this research.

4. SCGH preparation

The SCG was baked at 60˚C for 48 hr. After baking, it was digested with 98% concentrated sulfuric acid using SCG to sulfuric acid ratio of 1:8 w/v. The mixture was gently stirred continuously for 30 min and during digestion, the SCG was allowed to cool. Then, sodium hydroxide (0.5 M) was added to adjust the pH to 7.0 (Sime et al., 2017). SCG were separated by filtering through a nylon fabric filter with a pore size of 15 µm and glass fiber filter Whatman® GF/C (0.45 µm), respectively. The SCGH was used for further experimentation.

5. *C. protothecoides* BUUC1601 cultivation using SCGH

Natural sea water was filtered with a 20 µm polyester fabric filter, then chlorine was added to a final concentration at 5 mg/L and aerate for 24 hr. The standard Gillard's F/2 medium was prepared by using seawater which was provided from the previous step. The F/2 medium was then autoclaved at 121°C and 15 psi for 20 min. *C. protothecoides* BUUC1601 was cultured in a 250 mL Erlenmeyer flask with 100 mL culture medium. Then divided into 5 experimental sets i.e., standard Gillard's F/2 culture medium, F/2+2.5% SCGH (v/v), F/2+5% SCGH, F/2+10% SCGH and F/2+15% SCGH, respectively. Inoculum of microalgae was added into the culture medium with 10% (v/v). The cultures were placed in a room at 26°C with 80 µM/m²/s fluorescent lamp for 16 days. Shaking the culture by hand was done once a day for 3 min.

6. Analytical techniques

6.1 Cell counting

The cell count of microalgae was determined using a hemocytometer. Then, cell density and specific growth rate (μ) were calculated by using Eq. 1 (Roleda et al., 2013).

$$\mu = \frac{\ln(N_2 - N_1)}{t_2 - t_1} \quad (1)$$

When N_1 and N_2 as cell density (cells/mL) at time t_1 and t_2 (day), respectively

6.2 Dry weight

C. protothecoides BUUC1601 was cultured for triplicate. Microalgae was grown in standard F/2 medium with a salinity at 30 psu and under constant light using cool-white, fluorescent lamps at 80 µM/m²/s for 12 days. Culture was collected for cell counting and dry weight analysis every 2 days. Biomass of microalgae was filtered on dried GF/C filter paper, then rinse with distilled water and kept at 105°C until a stable weight (Ratomski & Hawrot-Paw, 2021). Filter paper was weighed and calculated of microalgae dry weight (g/L). XY-distributed graph was created, which X-axis=cell density (cells/mL) and Y-axis=microalgae dry weight (g/L). A linear equation was created as y=0.0058x +0.3992 (R^2=0.9529) for assessed dry weight in this experiment. Biomass productivity (*P*) in g/L/day was calculated using Eq. 2 (Ratha et al., 2016).

$$P = \frac{(N_2 - N_1)}{t_2 - t_1} \quad (2)$$

When N_1 and N_2 as microalgae dry weight at time t_1 and t_2, respectively

6.3 Total sugar content in the culture medium

The phenol-sulfuric acid method (Dubois et al., 1956) was used to determine total sugar content in the culture medium. First, the culture medium was filtered through GF/C filter paper. Then, 5.0 mL of filtered culture medium was pipetted and 5.2 mL concentred sulfuric acid and 0.5 mL of 5% Phenol were added. Then mixed to be homogeneous and incubated in a fume hood for 30 min. The absorbance was measured with a wavelength at 490 nm and calculated for the total sugar content (mg/L) by using the linear equation from the standard curve of the glucose solution.

6.4 Proximate composition analysis

The AOAC (1997) method was used to determine the proximate composition of SCG. The moisture content was determined by drying SCG in an oven at 105°C for 6 hr. The ash content was estimated by heating SCG in a muffle furnace at 550°C for 6 hr. Protein was determined

using the Kjeldahl's method. Lipids were extracted from SCG using petroleum ether and conducted in a Soxhlet extractor. The fiber content was analyzed using SCG that had been extracted oil. The grounds were boiled with 1.25% sulfuric acid and washed with boiled water, then boiled with 1.25% sodium hydroxide and washed again with boiled water. The mixture was filtered through a plankton net and the residue was baked at 100°C for 2 hr and then burned at 550°C for 5 hr. For carbohydrates, it was calculated using Eq. 3:

Carbohydrate (%) = 100 – ((Moisture (%) + Protein (%) + Lipid (%) + Fiber (%) + Ash (%)) (3)

6.5 Lipid extraction

At day 16 of the experiment, microalgae were collected by centrifugation at 10,000 rpm for 5 min. Next, it was important to discard the culture medium and rinse again with distilled water. Cell was dried by lyophilization method. The 50 mg of dried cells were extracted for lipids according to the method of Folch et al. (1957) by using chloroform: methanol (2:1). The lipid solution was evaporated by a rotary evaporator to eliminate mixed solvents. Next, they were sprayed with nitrogen gas, weighed for lipid determination and reported in % in dry weight.

6.6 Fatty acid analysis

Lipid extracted was dissolved with solvent, chloroform: methanol mixed with butylated hydroxytoluene (BHT) and transesterification by modified Christie's (2003) method. The solution was evaporated and dried with nitrogen gas, and the fatty acids were dissolved with 1 mL of n-hexane. The fatty acid solution was analyzed by gas chromatography (model 7890A, Agilent) with Flame Ionization Detector (FID), column model 19091N-133, HP-INNOWAX (Agilent). One µL sample was injected in split (5:1), flow rate of helium gas 1.1 mL/min, injection point temperature 240°C and detector temperature 260°C, which started at 35°C and maintained for 0.5 min. The temperature was then increased to 170°C at a rate of 3°C/min, maintained for 3 min, and the temperature was increased to 240°C at a rate of 2°C/min, maintained for 3 min. The fatty acid classification was compared with the retention time of Standardized Fatty Acids 37 Component FAME Mix (Supelco).

7. Statistical analysis

The average specific growth, cell yield, lipid yield of microalgae, and total sugar content in each experimental unit were calculated. The data were then subjected to One-Way Analysis of Variance (One-Way ANOVA) to analyze variance and compared differences between the means of experimental sets using Duncan's tests at a 99% confidence level. The statistical analysis was conducted using SPSS software (version 15; SPSS Inc.; Chicago, IL, USA).

Results and discussion

1. SCG and SCGH

The moisture content of SCG in the experiment was 6.90±0.03%. While the dry SCG contained 58.04% of carbohydrates, followed by fiber and protein at 19.12 and 13.94% in dry weight, respectively. The percentage of ash and lipid content was only 1.35 and 0.66%, respectively (Table 1). Dried SCG was digested using concentrated sulfuric acid at a 1:8 ratio while stirring constantly. Cooling was employed during digestion to reduce the severity of the reaction. Then, the pH value was adjusted to 7.0 and large particles were filtered to obtain SCGH. The total sugar content of SCGH was 69.21±1.94 mg/L.

SCG containing carbohydrates at 58.04% of dry weight were digested with strong acids, producing sugar products. Mussatto et al. (2011) reported that SCG contains approximately 50% of polysaccharides in dry weight. When SCG was digested by hydrolysis with sulfuric acid, it was found that SCGH consisted of mannose, galactose and arabinose at 21.2%, 13.8% and 1.7% by weight, respectively. The result suggests that SCGH may be able to be used as an organic carbon source for microalgae cultivation such as *Chlorella* and *Spirulina.* Microalgae can use organic carbon together with their food production from photosynthetic or mixotrophic (Melo et al., 2018). Therefore, in the mixotrophic cultivation, agricultural waste or wastewater from the processing of agricultural products in both solid and liquid form were used as ingredients of culture medium (Silkina et al., 2019).

Table 1 Chemical composition in SCG (mean percent ± SD) used in this research

Chemical composition	% Dry weight
Moisture content	6.90±0.03
Protein	13.94±0.18
Lipid	0.66±0.01
Carbohydrate	58.04±0.25
Ash	1.35±0.01
Fiber	19.12±0.08

2. *C. protothecoides* BUUC1601 Growth

Fig. 1 below, indicates that the use of SCGH in the culture medium had an effect on microalgae growth similar to the standard F/2 medium in autotrophic growth conditions (without SCGH). The marine green microalgae, *C. protothecoides* BUUC1601 was grown in F/2 culture medium with varying concentrations of SCGH. It was no significant difference in the specific growth rate, maximum cell density and biomass productivity ($p > 0.05$). With mixotrophic growth, *Chlorella* cultured with F/2 medium mixed with SCGH, had a growth efficiency and biomass yield similar to a cell that was cultured with F/2 medium. The specific growth rates ranged from 0.87 to 1.12 day^{-1} and biomass yields ranged from 0.05 to 0.08 g DW/L/day ($p > 0.05$) (Table 2). When *Chlorella* was cultured in F/2 medium without the addition of SCGH, its yield was slightly higher than that of *Chlorella* cultured using SCGH. This may be due to the F/2 medium containing a sufficient nitrogen source (in the form of nitrate) for their growth. Therefore, *Chlorella* can be used to generate new cells, which the accumulation of metabolites is reduced (Rios et al., 2016). The F/2 medium with SCGH had a higher source of organic carbon in the form of sugar than the F/2 medium without SCGH (Fig. 2), but nitrogen or nitrate in culture medium will be diluted. In addition, the preparation of SCGH by using strong acid digestion and adjusting the pH with alkali to neutralize the pH of SCGH effects the other nitrogen sources such as losing the free amino acid in the spent coffee grounds. Therefore, it may have an effect on C:N ratio in the culture medium to change. Silaban et al. (2014) reported that the mixotrophic culture of *Chlorella vulgaris* with a C:N ratio at 15:1 had a good result on their growth. However, when considering the biomass yield of microalgae, it indicated that microalgae cultivation by F/2 medium with the addition of SCGH in the range of 2.5-15% can produce biomass of *C. protothecoides* BUUC1601.

The results of this experiment showed that the sugar content in SCGH did not have any significant effect on the growth of *C. protothecoides* BUUC1601. However, a decrease in the total sugar content was observed in all experimental units (Fig. 2). Therefore, adding organic carbon to culture medium may also inhibit microalgae growth as well (Rios et al., 2016). The F/2 medium containing SCGH ranged in color from pale yellow to light brown, depending on the amount of SCGH added. This variation in color is due to the presence of tannins in SCGH, which may also cause the Maillard reaction during coffee roasting. It is important to note that the color of coffee beans can vary based on the specific strain of coffee, as well as the temperature and duration of roasting (Hariyadi et al., 2020). Therefore, if high concentration of SCGH is added to the culture medium, the color of the culture media darkens. It may have an effect on light transmittance of medium lower than the F/2 medium without SCGH and may have an effect on decreasing the photosynthetic efficiency.

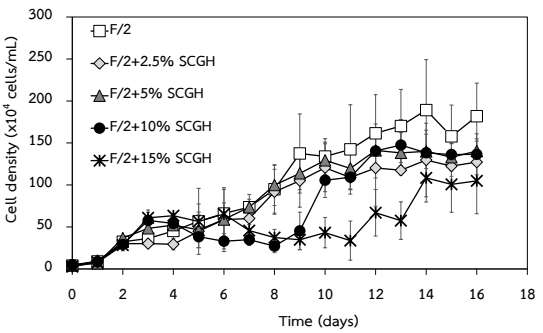

Fig. 1 Growth profiles of *C. protothecoides* BUUC1601 in each experimental unit during 16 days of culture (error bars = ± SD)

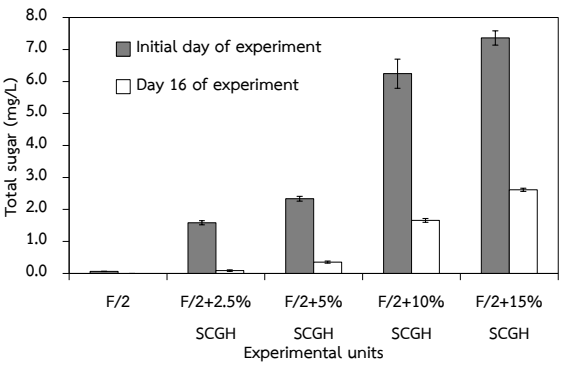

Fig. 2 Total sugar content in the culture of *C. protothecoides* BUUC1601 at initial day and the end of experiment (error bars = ± SD)

Table 2 Specific growth rate, maximum cell density and biomass productivity of *C. protothecoides* BUUC1601 (mean ± SD) cultured in F/2 culture medium supplied with different SCGH concentrations

Experimental unit	Specific growth rate^{ns} (day^{-1})	Maximum dry weight^{ns} (g DW/L)	Maximum biomass productivity^{ns} (g DW/L/day)
F/2	1.05±0.27	1.50±0.35	0.08±0.02
F/2+2.5% SCGH	1.12±0.07	1.19±0.20	0.06±0.02
F/2+5% SCGH	1.02±0.1	1.41±0.15	0.08±0.01
F/2+10% SCGH	0.87±0.20	1.26±0.17	0.06±0.01
F/2+15% SCGH	1.08±0.18	1.08±0.13	0.05±0.01

Remark: ns means no significant difference at $p > 0.05$

3. Lipid content and fatty acid composition in *C. protothecoides* BUUC1601

C. protothecoides BUUC1601 were grown in a stationary phase between days 14-16. The color of microalgae which was cultured in F/2 medium without SCGH was still green. Whereas, the microalgae cultured with F/2 medium with SCGH turned a yellowish green-yellow. The microalgae cultivated with F/2 medium by adding 15% SCGH (v/v) was yellower than the other experimental units (Fig. 3). When the cells were collected for lipid extraction, it was found that the addition of 15% SCGH had a highest lipid accumulation at 66.03±5.08% in dry weight ($p < 0.05$) (Table 3). It was shown that the microalgae culture with F/2 medium with 2.5-15% SCGH added had a higher lipid content than in F/2 medium without SCGH with 1.83 times, 2.45 times, 2.51 times and 2.89 times, respectively. Lizzul et al. (2018) reported that *C. sorokiniana* cultured in an autotrophic condition without added organic carbon had a lipid content range from 18–22%, which was similar to that of *C. protothecoides* BUUC1601 in this experiment (22.83%).

The SCGH contains various types of organic carbon such as fats, fatty acids and carbohydrates (Cruz-Lopes et al., 2017; Fei et al., 2015), which can serve as precursors for lipid production by microalgae. However, in this study, it was found that the addition of SCGH did not promote microalgae growth but changed their color from green to yellow (Fig. 3). This indicates that the microalgae may be under stress or have a low nitrogen content. SCGH contains a significant amount of organic carbon, but there is a limited amount of nitrogen that may have been lost during the process of preparing SCGH. This can affect microalgae by reducing protein production, stopping cell regeneration and increasing the accumulation of metabolites in the form of lipids or carbohydrates (Krzemin´ska et al., 2015; Ferreira et al., 2009). In addition, Converti et al. (2009) reported that factors such as inappropriate light intensity or lack of nutrients in culture media, especially nitrogen, can lead to increased lipid accumulation in microalgae. Therefore, due to limited nitrogen source *C. protothecoides* BUUC1601 was able to accumulate more lipids under the conditions of this study.

Fig. 3 Color of *C. protothecoides* BUUC1601 at the end of experiment

Table 3 Lipid content in *C. protothecoides* BUUC1601 (mean percent ± SD) cultured with different SCGH concentrations

Experimental unit	Lipid content (% in dry weight)
F/2	22.83±8.31^{c}
F/2+2.5% SCGH	41.86±10.19^{b}
F/2+5% SCGH	55.84±4.22^{ab}
F/2+10% SCGH	57.27±2.51^{ab}
F/2+15% SCGH	66.03±5.08^{a}

Remark: Values followed by different letters denote significant difference at $p < 0.05$

C. protothecoides BUUC1601 is abundant in saturated fatty acids such as myristic acid (C14:0), C15:0, palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0). When compared to cells cultured in F/2 medium without SCGH, those cultured with SCGH exhibited significantly lower amounts of C16:0 ($p < 0.05$), which accounted for up to 16.87% of total fatty acids. Ötleo & Pir (2001) reported that *Chlorella* contains palmitic acid ranging from 14.42% to 17.22%. Palmitic acid is a precursor for the synthesis of both saturated and unsaturated fatty acids and serves as the primary component of biodiesel for fuel energy production.

C. protothecoides BUUC1601 cultured with SCGH exhibited significantly higher levels of the total monounsaturated fatty acids compared to cells cultured in F/2 medium without SCGH (Table 4). The addition of SCGH at levels of 2.5-15% did not have a significant effect on the accumulation of polyunsaturated fatty acids ($p > 0.05$). Cells contained more total monounsaturated fatty acids (MUFAs) than polyunsaturated fatty acids, including myristoleic acid (C14:1), ginkgolic acid (C15:1), palmitoleic acid (C16:1) and oleic acid (C18:1n9). Oleic acid was the most abundant monounsaturated fatty acid in *C. protothecoides* BUUC1601 and in the genus *Chlorella* (Ferreira et al., 2017; Isleten-Hosoglu et al., 2012). Moreover, cells cultured in F/2 medium with SCGH (2.5-15%) had higher levels of C18:1n9 (c+t) up to 33.84-38.21% of total fatty acids, compared to cells cultured in F/2 medium without SCGH ($p<0.05$). Ferreira et al. (2017) reported that microalgae cultured under heterotrophic

conditions with organic carbon added to the medium and incubated in the dark accumulated up to 35.1% oleic acid. Although oleic acid is a non-essential fatty acid, it is an important energy fuel (Deshmukh et al., 2019).

The polyunsaturated fatty acid content of *C. protothecoides* BUUC1601 cultured with SCGH was significantly higher than cells cultured in F/2 medium without SCGH ($p < 0.05$), with a range of 32.40-34.62% of total fatty acids. Linoleic acid (C18:2n-6) was the most abundant polyunsaturated fatty acid, followed by gamma-linolenic acid (C18:3n6) and alpha-linolenic acid (C18:3n3) and the addition of SCGH had the greatest impact on the accumulation of linoleic acid, which accounted for 25.99-27.20% of total fatty acids. Cheng et al. (2013) also reported that *C. protothecoides* had high levels of C18:2 and C18:3. These fatty acids are essential for human health, with potential benefits including reducing the risk of heart disease, preventing cancer, arthritis, high blood pressure and diabetes (Kaur et al., 2014). They are also important for aquaculture, as Jardine et al. (2020) found that linoleic acid and alpha-linolenic acid can improve fish growth and survival rate. However, they are sensitive to oxidation reactions, which may affect the oxidation stability in biodiesel production.

The results of this experiment demonstrated that the addition of SCGH to F/2 medium at 2.5-15% could increase the content of certain unsaturated fatty acids in *C. protothecoides* BUUC1601, particularly oleic acid, linoleic acid, gamma-linolenic acid and alpha-linolenic acid. This may be attributed to the fact that coffee is a rich source of fatty acids. Figueiredo et al. (2015) reported that *Coffea arabica* L. cultivated in Brazil contained C18:1 in the range of 0.37-10.73%, C18:2 in the range of 36.68-41.50% and C18:3 at 0-1.64%. Therefore, the addition of SCGH to the culture medium may have contributed to the increased accumulation of fatty acids in the microalgae.

Conclusion

SCGH prepared from spent coffee grounds can be added to the F/2 medium for *C. protothecoides* BUUC1601 culture under mixotrophic conditions. SCGH helps the microalgae accumulate more lipids than the standard F/2 medium without SCGH (under autotrophic conditions). Adding SCGH at 15% (v/v) can increase the amount of lipid up to 66.03% in dry weight, which is 2.89 times more than microalgae cultured without SCGH.

Table 4 Fatty acid content (expressed as percentage of total fatty acids) in *C. protothecoides* BUUC1601 cultured with different SCGH concentrations

Fatty acid	F/2	F/2+2.5% SCGH	F/2+5% SCGH	F/2+10% SCGH	F/2+15% SCGH
SFAs					
C14:0	2.62±2.99^{a}	2.83±0.84^{a}	1.84±0.40^{a}	3.32±0.23^{a}	2.95±0.42^{a}
C15:0	1.53±1.73^{a}	1.06±0.42^{a}	0.62±0.15^{a}	0.71±0.41^{a}	0.87±0.20^{a}
C16:0	16.87±7.14^{a}	4.27±0.60^{b}	3.21±0.17^{b}	4.07±0.69^{b}	4.42±0.33^{b}
C17:0	3.01±1.08^{a}	3.79±0.21^{a}	3.35±0.28^{a}	3.21±0.14^{a}	3.28±0.05^{a}
C18:0	6.90±2.53^{a}	7.19±0.24^{a}	11.35±3.90^{a}	8.14±0.16^{a}	9.03±0.20^{a}
C20:0	0.74±0.84^{a}	0.83±0.50^{a}	0.52±0.07^{a}	0.65±0.05^{a}	0.61±0.06^{a}
C24:0	1.85±4.53	N. D.	N. D.	N. D.	N. D.
MUFAs					
C14:1	5.00±4.67^{a}	1.08±0.85^{a}	1.8±0.67^{b}	0.78±0.07^{a}	0.68±0.08^{a}
C15:1	3.19±4.04^{a}	1.82±0.92^{a}	1.25±0.16^{a}	1.67±0.57^{a}	1.67±0.21^{a}
C16:1	4.61±1.61^{b}	6.85±2.78^{a}	4.64±0.55^{ab}	5.09±0.07^{ab}	4.82±0.27^{ab}
C17:1	N. D.	0.58±0.08^{a}	0.41±0.13^{a}	0.4±0.05^{a}	0.37±0.07^{a}
C18:1n9c	21.62±5.45^{b}	31.70±1.20^{a}	34.46±1.21^{a}	34.27±0.41^{a}	32.63±0.27^{a}
C18:1n9t	2.08±0.47^{b}	2.84±1.40^{ab}	3.62±0.27^{a}	3.94±0.09^{a}	3.71±0.10^{a}
C20:1	1.14±2.78^{a}	1.30±1.63^{a}	0.35±0.06^{a}	N. D.	0.35±0.07^{a}
C22:1n9	1.00±2.45	N. D.	N. D.	N. D.	N. D.
PUFAs					
C18:2n6	17.61±5.18^{b}	27.18±0.90^{a}	25.99±1.34^{a}	26.93±0.61^{a}	27.20±0.33^{a}
C18:3n6	N. D.	6.77±0.21^{a}	6.03±0.22^{a}	6.19±0.15^{a}	7.01±0.33^{a}
C18:3n3	4.20±1.98^{a}	0.49±0.24^{b}	0.38±0.06^{b}	0.64±0.24^{b}	0.41±0.10^{b}
C20:2	2.93±7.17	N. D.	N. D.	N. D.	N. D.
C22:2	2.55±6.24	N. D.	N. D.	N. D.	N. D.
C22:6n3	0.56±1.36	N. D.	N. D.	N. D.	N. D.
Total SFAs	33.52±0.90^{a}	19.97±0.01^{b}	20.89±0.827^{b}	20.10±0.17^{b}	21.16±0.06^{b}
Total MUFAs	38.64±0.42^{b}	46.17±0.80^{a}	46.53±0.41^{a}	46.15±0.01^{a}	44.23±0.08^{a}
Total PUFAs	27.83±0.48b	34.44±0.20a	32.40±0.69a	33.76±0.42a	34.62±0.24a

Remark: N.D: Not detected, SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFAs: Polyunsaturated fatty acids, Values in each row followed by different letters denote significant difference at $p < 0.05$

However, adding SCGH to the medium has an effect on the monounsaturated fatty acid content, increasing it to 46.15-46.53% and the polyunsaturated fatty acid content to 32.40-34.62% of total fatty acids. Oleic acid was found to be more than 30% of total fatty acids, while omega-6 fatty acids such as linoleic acid were found at 25.99-27.20% and gamma-linolenic acid at 6.03-7.01% of total fatty acids. The research found that cultivating the microalgae with the addition of SCGH at 2.5-15% can produce lipid in *C. protothecoides* BUUC1601, indicating its potential use in both aquaculture and as a functional food supplement.

Acknowledgements

The authors wish to extend our appreciation to Hidden Gem Café, Chanthaburi, Thailand for providing us with the spent coffee ground samples and to the Marine Technology Research Center, Faculty of Marine Technology, Burapha University for providing the use of laboratories and scientific instruments.

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Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Effect of Herbal Plant Extracts on Inhibition of Pathogenic Bacteria in Nile Tilapia (*Orechromis niloticus*)

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Article info

Article history:
Received : 6 March 2023
Revised : 19 April 2023
Accepted : 23 April 2023

Keywords:
Plant extracts, Antibacterial activity, Nile tilapia, Fish pathogens

Abstract

Herbal plant extracts could be an alternative treatment tilapia fish for diseases. This research was carried out to evaluate the antimicrobial potential of several plant extracts against fish pathogenic bacteria isolated from diseased or health Nile tilapia. This study selected twenty plant extracts to determine anti-bacterial activity by agar well diffusion method on trypticase soy agar (TSA).Aqueous ethanolic extracts of herbs were tested for activity against three fish pathogenic bacteria, including *Aeromonas hydrophila, Streptococcus agalactiae* and *Edwardsiella ictaluri.* Effective herbal plant extracts was selected to investigate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values against representative bacterial stains by broth dilution method. The results showed that *Terminalia chebula, Acacia catechu, Glycyrrhiza glabra, Psidium guajava, Houttuynia cordata, Piper sarmentosum* and *Garcinia mangostana* extracts could inhibit all strains of the test bacteria. However, inhibitory zone effects of *A. catechu* extracts showed the best activity against *A.hydrophila, S.agalactiae* and *E.ictaluri* at 22.66±0.29 mm, 24.33±0.09 mm and 21.33±0.34 mm, respectively when compared with other herb extracts, with the zone of inhibition ranging from 8.33±0.84 to 19.33±0.04 mm Generally, MIC and MBC concentration values of *A. catechu* extracts that ranged between 6.5 and 12.5 mg/mL of aqueous plant extracts for all pathogens tested. The results suggest that herbal plant extracts showing several biological activities may be potent inhibitors as a natural anti-bacterial in Nile tilapia.The findings suggest that herbal plant extracts could be a potential alternative to fish disease therapy.

Introduction

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from herbal plants. Many regions of Thailand have a diverse assortment of plants. Although some indigenous plants are uncommon, their potential bioactivities should not be overlooked

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(Thummajitasakul et al., 2014). Various medicinal properties have been attributed to different components of the plant. Herbs that have been used for centuries to treat bacterial infections were reported to have a high number of phytochemicals with versatile and novel structures. Herbs are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids, steroids and phenolic compounds, shown in vitro to have antibacterial effects (Chahal et al., 2016; Dorman & Deans, 2000). A natural supply for the food business is aquaculture. An increase in demand for aquaculture production for food supply forces the fisheries to increase fish population densities. As a result, poor aquaculture sanitation, a rise in fish populations, and immune system suppression make people more susceptible to bacterial and viral illnesses as well as other diseases (Cabello, 2006). Antibiotics and chemical therapies are currently common treatments for bacterial infection. However, there are restrictions on use, side effects, low efficacy and antibiotic-resistant bacteria with these treatments (Zheng et al., 2000). Utilizing various plant extracts to control pathogen infection in fish is an alternative therapeutic and environmentally friendly method. This is due to the nature of plant-derived extracts which are non-harmful to the environment, water-degradable, ease of preparing procedures, safe in an aquatic ecosystem, low in cost, a good potential source against fish pathogenic bacteria and does not cause a resistant effect on fish, thereby suitable for a sustainable aquaculture (Defoirdt et al., 2011). Due to the fact that many of these promising natural medicinal plants grow in Thailand, the use of plant extract to prevent bacterial infection in fish has a high potential for implementation. Bacterial pathogens are among the emerging diseases and are a major threat to fish production. Some herbs have been reported to have antimicrobial activity against a variety of pathogenic bacteria and have been used as traditional medicines for tilapia fish treatment (Abutbul et al., 2004; Yin et al., 2006).

Oreochromis niloticus also known as Nile tilapia is one of the most significant species in freshwater aquaculture. Due to its benefits of easy breeding, specific disease resistance, rapid development and high marketability, it has become the world's second-most extensively farmed freshwater fish species (Etyemez & Balcazar, 2016). Although Tilapia aquaculture has grown rapidly, it also encounters considerable difficulties due to diseases brought on by *Streptococcus agalactiae, Edwardsiella ictaluri, Aeromonas hydrophila, Vibrio* sp., *Pseudomonas* sp. and *Flavobacterium* sp. Bacterial infection in particular can lead to major issues for the fish, such as septicemia, exophthalmia, corneal opacity and various swimming abnormalities, causing mortality and economic losses in aquaculture (Guo et al., 2019). *A. hydrophila* and *E. ictaluri* are short, gram-negative bacteria found to infect freshwater fish. Fish infected with *A. hydrophila* bacteria resemble having ulcers in their external organs under stress conditions, bacteremia, enteritis, respiratory tract failure, dysentery and motile Aeromonas septicemia (MAS). MAS is stress-related and conditions such as poor water quality, overcrowding and rough handling make fish more susceptible to the bacteria. Fish with MAS infection usually have bulging eyes, swollen abdomens and exhibit small pinpoint hemorrhages at the base of the fins or on the skin. Internal signs include abdominal fluid, enlarged liver and spleen, and dilated and fluid-filled intestines (Yu et al., 2007). The infected fish *E. ictaluri* may exhibit internal organs with excess water and a severe chronic inflammatory infiltrate is observed in the spleen, head kidney and liver, with multifocal areas of necrosis and granuloma formation (Soto et al., 2012). *S. agalactiae* is a gram-positive prominent and ubiquitous pathogen in aquaculture, resulting in annually world-wide significant morbidity (generally 20–30%) and mortality (over 95% of diseased fish), particularly in the tilapia aquaculture business (Su et al., 2017). Disease outbreaks have a negative impact on mortality rates and productivity efficiency, resulting in significant economic losses for fish producers (Hatha et al., 2005). Antibiotics are still used as current treatments for bacterial infections in Asian aquaculture. However, considering the inherent negative effects of antibiotics, other alternative antimicrobials from plant origins are increasingly used in aquaculture. Temu kunci (*Boesenbergia pandurata*), terong asam (*Solanum ferox*) and lempuyang (*Zingiber zerumbet*) extracts also have antibacterial properties that might inhibit the development of *A. hydrophila* and *Pseudomonas* sp. bacteria, which are the Nile tilapia's common pathogens (Hardi et al., 2017). Furthermore, the extract of *Rosmarinus officinalis* have been reported to treat the bacteria infection caused by *Streptococcus iniae* in Nile tilapia (Abutbul et al., 2004; Zilberg, et al., 2010). According to Yin et al. (2006), investigated the results of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) were also found to have therapeutic effect on diseased tilapia. Meanwhile, application of solo garlic (*Allium sativum*), garlic chive (*Allium tuberosum*)

and betel leaves (*Piper betle*) extract on bacterial pathogens in aquaculture and a challenge of Nile tilapia, *O. niloticus* with *S. agalactiae* were presented (Ataguba et al., 2018). Many substances with antibacterial activity can be synthesized by a variety of plant species. These qualities have been attributed to extracts of several Thailand-found plants. Many herb extracts were investigated on their antimicrobial activity against some fish pathogenic bacteria. Conversely, there haven't been any prior studies analyzing this characteristic for several of the plant species included in this present research. Agar diffusion techniques are used widely to assay plant extracts for antimicrobial activity. The zone of inhibition, as assessed by the agar well diffusion technique, differed depending on the plant extracts. The findings from the agar diffusion assay plates were in fair correlation with those from the MIC testing. Therefore, considering their antibacterial qualities, accessibility and diminished concerns about antimicrobial resistance and antibiotic residues in aquaculture products, the use of herbal additives seems to be a sensible and financially advantageous strategy for aquaculture producers (Anka et al., 2013).

The present work investigated the in vitro antimicrobial effects of ethanolic extract of twenty herbal plants against three fish pathogenic bacteria strains, such as *A.hydrophila, S. agalactiae* and *E. ictaluri* which are incriminated in different diseases of Nile tilapia, one of Thailand's most widely cultivated fish species and may serve as a substitute for synthetic antibiotics in fish raised in farms.

Materials and methods

1. Bacterial preparation

Pathogenic bacteria used in the treatment were two types of Gram-negative rod (*A. hydrophila* and *E. ictaluri*) and one type of Gram-positive coccus (*S. agalactiae*). They were obtained and well identified from Charoen Pokphand Foods (CPF) Public Company Limited, Mueang Samut Sakhon District, Samut Sakhon Province, Thailand. All the cultures were maintained and sub-cultured on Trypticase Soy Agar, TSA, (DIFCO®) slants at 37°C overnight. Bacteria were cultured in Trypticase Soy Broth, TSB, (DIFCO®) media at 37°C, for 24 hr until they reached a log-phase in their growth. The cultures were then centrifuged for 10 min at 1000xg. A phosphate-buffered saline (PBS) solution was used to wash the pellet three times while removing the supernatant. For challenge test, the bacteria suspensions were diluted using sterilized distilled water and standardized using spectrophotometer at 600 nm to an optical density (OD) of 0.1, corresponding to 10^7 CFU/ml (Hardi et al., 2017).

2. Preparation of plant extracts

In the investigation, the whole plant of *Acacia catechu* (wood/bark), *Acanthus ebracteatus* (root/stem/leaf), *Allium ascalonicum* (bulb), *Artemisia annua* (root/stem/leaf), *Artemisia lactiflora* (stem/leaf), *Bauhinia malabarica* (leaf), *Cassia fistula* (fruit; seed aril), *Clinacanthus nutans* (root/stem/leaf), *Eclipta prostrata* (root/stem/leaf), *Garcinia mangostana* (peel), *Glycyrrhiza glabra* (root), *Gynostemma pentaphyllum* (root/stem/leaf), *Houttuynia cordata* (stem/leaf), *Piper retrofractum* (fruit), *Piper ribesiodes* (stem), *Piper sarmentosum* (root), *Psidium guajava* (leaf), *Rhinacanthus nasutus* (root/stem/leaf), *Terminalia chebula* (fruit) and *Tinospora crispa* (stem) were obtained from a local market and grown naturally in Thailand. The gathered plants were taxonomically recognized and verified. Each herbal plant was cleaned with distillated water, dried for 48 hr at 40°C and grinded into powder. To the extraction, 300g of dried powdered plant sample was suspended in 600 mL of ethanol 14.5% at 80°C for 24 hr with constant stirring. After heating, the extract was filtered by 5-micron paper filter and evaporated solvent at 80°C into concentrated liquid. Dried in trey dryer under 50°C with constant air flow to obtain extract powder. After complete solvent evaporation, extracts were dissolved in 10% dimethyl sulphoxide (DMSO) to a final concentration of 100 mg/mL and stored at 5°C in labelled sterile screwcapped bottles for further use (Castro et al. 2008).

3. Antibacterial activity assay

The standard agar well diffusion technique was used to evaluate the antibacterial activity of solvent extracts (Valgas et al., 2007). Inoculum containing 10^7 CFU/mL of each bacterial culture to be tested was swabbed on trypticase soy agar (TSA) plates, subsequently wells of 6 mm diameter were punched using sterile cork borer into the agar medium and filled with 50 µL (100 mg/mL) of plant extract and allowed to diffuse at room temperature for 2 hr. The plates were then incubated for 24 hr at 37°C in the upright position. As negative controls, wells containing the same amount of DMSO (10%) were used. After incubation, the diameters of the growth inhibition zones were measured in millimeter (mm). Three replicates were carried out for each extract against each of the test organism and the average values were

recorded. Data were expressed as mean±standard deviation. Four classifications were made based on the zone of inhibition: weak (<5 mm), moderate (5-10 mm), strong (10-20 mm) and very strong (>20 mm) (Dewi & Mardhiyani, 2021).

4. Bacteriostatic and bactericidal activity

The bacteriostatic or MIC is generally defined as the lowest concentration of a given antimicrobial that prevents growth of a microorganism after a specified incubation period. Based on the preliminary screening, ethanol extracts with high antibacterial activity were examined and further tested to determine the MIC for each bacterial sample. By using a broth dilution approach, the MIC of these extracts was determined. The stocks of 100 mg/mL of the extracts were resuspended in 10% DMSO to produce serial twofold dilutions in the range of 0.1-100 mg/mL. The final concentration of each plant extract was 50, 25, 12.5, 6.5, 3.1, 1.6, 0.8, 0.4, 0.2 and 0.1 mg/mL. Briefly, 1 mL of TSB and 1 mL of graded doses of crude extract were added to each test tube. After that, 1 mL of suspended bacterial suspension (1×10^5 CFU/mL) was inoculated to these test tubes followed by incubation at 37°C for 24 hr. After incubation, a spectrophotometer set to 600 nm was used to measure turbidity as the lowest concentration of plant extracts that prevented the bacterial isolates in the test tubes from growing. The MIC value was established and recorded as being the lowest concentration at which no turbidity was detected. Two test tubes including the extract with no bacteria and bacteria with no extract were used as negative control and positive control, respectively. All samples were tested in triplicates. To evaluate the Minimum bactericidal concentration (MBC), the tubes that did not exhibit any apparent growth (clear solution) were collected from each tube using an inoculation loop and re-cultured on TSA. The lowest concentration at which there was no apparent bacterial growth was recorded as the MBC values after incubation. The assays were done in triplicate (Elisha et al.,2017; Aiyegoro et al., 2009).

Results and discussion

In this study, we investigated multiple herbs for antibacterial efficacy against bacterial infections in warm water fish such as tilapia (*Oreochromis niloticus*). In Table 1, the outcomes of the agar diffusion assay used to screen for the presence of antimicrobials are displayed. According to the findings, the test microorganisms' growth was inhibited to varied degrees by plant extracts from plants at a concentration of 5 mg. Twenty ethanolic extracts were shown to have antibacterial action against at least one strain tested. The organism *A. hydrophila* was the most vulnerable and 14 plant extracts inhibited it. *S.agalactiae* and *E. ictaluri* were inhibited by eleven and eight plant extracts, respectively. The efficacy of the antimicrobial agents in the ethanolic extract of plants was assessed using the MIC and MBC assays. In diagnostic laboratories, determining the MIC and MBC is essential because it assists with identifying if a microbe is resistant to an antimicrobial agent and it keeps track of the activity of new antimicrobial agents. The results range between 6.5 and 50 mg/mL for the MIC and MBC of the extract against all pathogenic bacteria tested and the results are presented in Table 2. There was a wide range of MIC and MBC values depending on the specific microbial strains being tested.

Table 1 Bacterial inhibition zone (mm) of plant extracts (5 mg) in agar diffusion assay

No.	Species	Inhibition zones (mm)		
		A. hydrophila	*S. agalactiae*	*E. ictaluri*
1	*Acacia catechu*	22.66±0.29	24.33±0.09	21.33±0.34
2	*Acanthus ebracteatus*	11.00±0.14	-	10.00±0.28
3	*Allium ascalonicum*	-	15.00±0.08	-
4	*Artemisia annua*	12.33±0.24	14.33±0.14	-
5	*Artemisia lactiflora*	13.33±0.81	13.00±0.08	-
6	*Bauhinia malabarica*	8.33±0.02	13.66±0.04	-
7	*Cassia fistula*	11.00±0.14	-	-
8	*Clinacanthus nutans*	10.00±0.03	-	15.33±1.12
9	*Eclipta prostrata*	-	-	-
10	*Garcinia mangostana*	9.33±0.09	9.33±0.09	10.00±0.03
11	*Glycyrrhiza glabra*	19.00±0.29	16.33±0.09	15.33±0.05
12	*Gynostemma pentaphyllum*	14.66±1.14	-	15.00±0.32
13	*Houttuynia cordata*	10.00±0.04	15.00±0.23	10.00±0.46
14	*Piper retrofractum*	-	-	-
15	*Piper ribesiodes*	-	-	-
16	*Piper sarmentosum*	11.00±0.14	12.00±0.17	10.00±0.87
17	*Psidium guajava*	8.33±0.84	9.00±0.14	15.00±0.09
18	*Rhinacanthus nasutus*	-	-	-
19	*Terminalia chebula*	12.00±0.28	12.00±0.54	19.33±0.04
20	*Tinospora crispa*	-	-	-

Remark: (-) Inhibition zone not observed

The ethanolic extract of four plants, as shown in Fig. 1, have antibacterial activity utilizing the agar well diffusion technique against three fish pathogenic bacteria. The results showed that *A. catechu, G. glabra, T. chebula, P. guajava, H.cordata, P. sarmentosum* and *G. mangostana* formed inhibition zones against all bacterial strains. Inhibitory effects of *A. catechu* extracts showed the best activity against *A. hydrophila, S. agalactiae* and *E. ictaluri* with inhibition zone at

Table 2 MIC and MBC of plant ethanolic extracts to selected fish bacterial pathogens

No.	Species	*A. hydrophila*		*S. agalactiae*		*E. ictaluri*	
		MIC values (mg/mL)	MBC values (mg/mL)	MIC values (mg/mL)	MBC values (mg/mL)	MIC values (mg/mL)	MBC values (mg/mL)
1	Acacia catechu	6.5	6.5	6.5	6.5	12.5	12.5
2	Acanthus ebracteatus	6.5	6.5	-	-	-	-
3	Allium ascalonicum	-	-	12.5	12.5	-	-
4	Artemisia annua	12.5	25.0	25.0	50.0	-	-
5	Artemisia lactiflora	25.0	50.0	25.0	50.0	-	-
6	Bauhinia malabarica	12.5	12.5	12.5	12.5	-	-
7	Cassia fistula	50.0	50.0	-	-	-	-
8	Clinacanthus nutans	12.5	25.0	-	-	-	-
9	Eclipta prostrata	-	-	-	-	-	-
10	Garcinia mangostana	25.0	50.0	6.5	6.5	12.5	12.5
11	Glycyrrhiza glabra	12.5	12.5	12.5	12.5	25.0	25.0
12	Gynostemma pentaphyllum	50.0	50.0	-	-	12.5	12.5
13	Houttuynia cordata	25.0	50.0	12.5	25.0	12.5	25.0
14	Piper retrofractum	-	-	-	-	-	-
15	Piper ribesiodes	-	-	-	-	-	-
16	Piper sarmentosum	12.5	12.5	12.5	12.5	12.5	12.5
17	Psidium guajava	25.0	25.0	25.0	25.0	12.5	12.5
18	Rhinacanthus nasutus	-	-	-	-	-	-
19	Terminalia chebula	12.5	12.5	6.5	6.5	6.5	6.5
20	Tinospora crispa	-	-	-	-	-	-

Remark: (-) MIC/MBC assay were excluded due to no inhibition somes observed (see table 1)

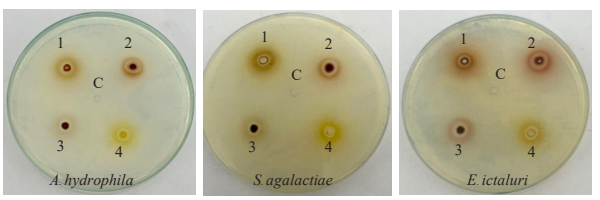

Fig 1. The inhibition zone (mm) of extracts of *T. chebula* (1), A. catechu (2), *P. guajava* (3) and *G. glabra* (4) against *A. hydrophila, S. agalactiae* and *E. ictaluri* at concentration of 5 mg (C) represent the negative control, 10% v/v DMSO

22.66±0.29 mm, 24.33±0.09 mm and 21.33±0.34 mm, respectively. *A. catechu*, commonly known as catechu, cachou and black cutch is a valuable medicinal plant and a crucial forest tree from an economic standpoint (Negi & Dave, 2010). This extract proved to be equally effective against both gram-positive and gram-negative bacteria. All the bacterial strains tested was found most susceptible with maximum inhibition by ethanolic extract producing zone of inhibition >20 mm (> 20 as very strong activity). MIC and MBC of this extract was 6.5 mg/mL against *A.hydrophila* and *S. agalactiae* while it was 12.5 mg/mL for *E. Ictalurid*, respectively. The results of the chemical research on *A. catechu* revealed that different parts had extremely high concentrations of flavonoids, tannins and phenolic substances. More specifically, this plant contains large amounts of catechin which serves as an antioxidant and antimicrobial agent (Singh et al., 2005). The extracts of the roots of *G. glabra* has shown magnificent antibacterial effect. Species of *G. glabra* (licorice or liquorice) also showed the capacity of inhibit the growth of *A. hydrophila, S. agalactiae* and *E. ictaluri* at 19.00±0.29 mm, 16.33±0.09 mm and 15.33±0.05 mm, presenting MIC and MBC values of 12.5 mg/mL, 12.5 mg/mL and 25.0 mg/mL respectively. The primary biomolecules of the roots include flavonoids, glycyrrhizinic acid (glycyrrhizin), glabridin, liqueurite and liqueurizine, which have potential antimicrobial (Mamedov & Egamberdieva, 2019). Recent research has discovered that consuming licorice roots can increase the non-specific immune system in Nile tilapia (Abdel-Tawwab & El-Araby, 2021). *T. chebula* showed inhibition zone of 12.00±0.28 mm against *A. hydrophila* and *S. agalactiae.* The highest mean diameter of inhibition zone of 19.33±0.04 mm against *E. ictaluri.,* MIC and MBC concentration values that ranged between 6.5 and 12.5 mg/mL for all pathogens tested. *T. chebula* fruit extracts have potent antibacterial activity against a number of gram-positive and gram-negative pathogenic bacteria. It is well recognized that this plant is a significant source of secondary metabolites, with hydrolysable tannins accounting for 33% of total phytoconstituents (which can range between 20-50%) and is responsible for antibacterial activity (Sharma et al., 2012). The inhibition zone diameters 8.33±0.84, 9.00±0.14, and 15.00±0.09 mm were obtained from the extract of *P. guajava* leaf against *A. hydrophila, S. agalactiae* and *E. ictalurid*, respectively. This extract had the lowest MIC and MBC (12.5 mg/mL) of *E. ictalurid*, while the highest value (25 mg/mL) of *A. hydrophila* and *S. agalactiae.* Guava is used to isolate flavonoids and saponins from leaves. These leaf extracts are known to have antimicrobial (Metwally et al., 2010). Extract from *G. mangostana* (mangosteen) pericarp was effective against *A. hydrophila, S. agalactiae* and *E. ictaluri* at inhibition zone diameter 9.33±0.09, 9.33±0.09 and 10.00±0.03 mm, respectively. The MIC and MBC for *A. hydrophila* and *S. agalactiae* were 25 mg/mL while it was 12.5 mg/mL for *E. ictalurid* respectively. According to phytochemical investigations, its active components belong to a group of xanthone derivatives such as α-, β- and γ-mangostin, gartinin, 1- and 3-isomangostin, etc. Among these, α-mangostin has the most potent antibacterial activity against both gram-positive and gram-negative bacteria. In our results

we found that *H. cordata* and *P. sarmentosum* extracts showed inhibition activity against *A. hydrophila, S. agalactiae* and *E. ictaluri.* The shoots of *H. cordata* are edible in China and Malaysia and have been used medicinally throughout Asia. According to reports, *H. cordata* extracts contains many constituents such as essential oil, flavonoids and other polyphenols, alkaloids, organic acid and fatty acid, sterols and microelements and have a variety of pharmacological activities including antibacterial (Kumar et al., 2014). In addition, phytochemical screening of the *P. sarmentosum* crude extract indicated the presence of tannins, flavonoids, alkaloids glycosides and anthraquinone (Fernandez et al., 2012).

The other antimicrobial active herb extracts, including produced zones of growth inhibition against *A. Hydrophila,* ranged between 8.33±0.84 and 11.66±1.14 mm. While *S. agalactiae* was inhibited with a range between 9.33±0.09 and 15.00±0.23 mm. and *E. ictalurid* was inhibited ranging between 10.00±0.03 and 15.33±0.05 mm. Moreover, these herb extracts had MIC values of 6.5 mg/mL (2 extracts), 12.5 mg/mL (6 extracts), 25.0 mg/ml (4 extracts) and 50.0 (2 extracts) against *A. hydrophila,* 6.5 mg/mL (3 extracts), 12.5 mg/mL (5 extracts) and 25.0 mg/mL (3 extracts) against *S. agalactiae.* and 6.5 mg/mL (1 extract), 12.5 mg/mL (4 extracts) and 25.0 mg/mL (1 extracts) against *E. ictaluri.* The outcomes for the MBC values matched those for the MIC values. Those presenting the MBC ranged from 6.5 mg/mL to 50 mg/mL. These herbs and their components, such as levamisole, alkaloids, flavonoids, essential oils, terpenes, organic acids, coumarins and lignans, have been shown to have potential antimicrobial effects in tests both in vitro and in vivo (Hardi et al., 2016). Herbs bring forth antibacterial activities by damaging cell walls, inhibiting nucleic acid and protein synthesis and increasing intracellular osmotic pressure (Liang et al., 2022). In addition, antibacterial chemicals have the ability to quickly damage the bacterial cell wall and cytoplasmic membrane resulting in cytoplasm leakage (Shan et al., 2007). In this research, the aqueous extract of *A. ebracteutus* inhibited the growth of *A. hydrophila.* It is a mangrove plant used in traditional medicine in Malaysia, Thailand and China. *A. ebracteatus* was also found to contain megastigmane, benzoxazinoid glycosides and aliphatic alcohol and it has been tested against several bacteria (Kanchanapoom et al., 2001). An antimicrobial property of shallot (*A. ascalonicum*) can effectively inhibit a wide range of pathogenic bacteria. According to Cowan (1999), the antibacterial mechanism of quercetin in shallot may be through membrane rupture and destruction of bacterial extracellular proteins by generating an irreversible complex. In the present study, *S. agalactiae* was inhibited with Shallot extracts of 15±0.08 mm. inhibition zone. Globally, there are around 500 species of *Artemisia* (Astraceae). The primary objective of this study was to evaluate their antimicrobial activities against fish bacterial strains of *Artemisia* spp. (*A. annua* and *A. lactiflora*). The outcomes showed that certain bacterial strains were successfully inhibited by the crude extract of these plants. Corresponding our data, *A. annua* extracts were previously shown to have strong bactericidal activity against *A. hydrophila* and *S. agalactiae,* suggesting that these plants contain bactericidal compounds beyond artemisinin (Soares et al., 2020). Along with artemisinin, *Artemisia* spp. also contains flavonoids and phenolic compounds like rosmarinic and chlorogenic acids (Gouveia & Castilho, 2013). From this research, the extracts of *B. malabarica* demonstrated promising activity against fish pathogenic bacteria *A. hydrophila* and *S. agalactiae.* In other research, using crude extracts of Bauhinia parts, promising activities were demonstrated, with substantial potential for antibacterial inhibition of the plant against a variety of bacteria (Neto et al., 2008). For this reason, the crude extracts of *Bauhinia* spp. presented saponins, tannins and alkaloids since this compound is known to have an antimicrobial effect against various microorganisms (Neto et al., 2008). Also considerable effects of *C. fistula* (*Leguminosae*) extracts against some fish pathogenic bacteria was observed. Consequently, the extracts inhibited *A. hydrophila* efficiently. According to Rizvi et al., (2009), this is the outcome of several substances in the aerial parts of *C. fistula*, such as flavonoids and polysaccharides. It can be found that *C. nutans* extracts showed activity against *A. hydrophila.* This medical herb has numerous chemical compounds that possess antibacterial activities such as, β-sitosterol, stigmasterol and flavonoids (Xie et al., 2015). The results of our experiment showed that *G. pentaphyllum* extracts against *A. hydrophila* and *E. ictalurid*. It is an edible plant used as a medicine in oriental countries. The bacterial activity of *G. pentaphyllum* has been reported. For instance, it has been shown that extract substances such as saponin were used to inhibit fungi and bacteria human diseases (Srichana et al., 2011).

The results of this study showed that the six herbs

(*A. ascalonicum, E. prostrata, E. prostrata, P. retrofractum, P. ribesiodes, R. nasutus* and *T. crispa*) did not inhibit *A. hydrophila,* while nine herbs (*A. ebracteatus, C. fistula, C. nutans, E. prostrata G. pentaphyllum P. retrofractum, P. ribesiodes, R. nasutus* and *T. crispa*) did not inhibit *S. agalactiae* and twelve herbs (*A. ebracteatus, A. ascalonicum, A. annua, A. lactiflora, B. malabarica, C. fistula, C. nutans, E. prostrata, P. retrofractum, P. ribesiodes, R. nasutus* and *T. crispa*) did not inhibit *E. ictaluri.* In this investigation, none of the extracts examined shown antibacterial efficacy against all test bacterial strains. Antibiotic-resistant bacterial strains have been observed in aquaculture systems. Possibly, the potentially deleterious impact of the extracts on the bacterial cells should be inhibited by the same mechanism engaged in antibiotic resistance. Nonetheless, some extracts were effective against pathogens, suggesting a potential alternative to fish disease therapy.

Conclusion

According to the study's findings, some herbs may have the ability to act as natural antimicrobial agents to prevent the growth fish pathogenic microorganisms. Of the 20 plants, crude ethanolic, i.e., extracts of seven, *A. catechu, G. glabra, T. chebula, P. guajava, H.cordata, P. Sarmentosum* and *G. mangostana* exhibited an inhibitory effect against all test bacterial strains, including *A. hydrophila, S. agalactiae* and *E. ictaluri.* The *A. catechu* showed high antimicrobial activity against all pathogens tested. No aqueous crude extract was found to inhibit any tested bacteria. The results indicated that different plant types significantly affected antibacterial activities. The extracts may be further investigated to identify and isolate the active components as a complementary therapy for the bacterial fish infections that are currently seen in Thailand fish farming.

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Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Formulation of Gelatin-Based Wheatgrass Leaf Juice Gummy Jellies with Antioxidant and the Analyses of Physicochemical and Texture Properties as Well as Evaluate the Nutritional Property of Selected Formulation

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Article info

Article history:
Received : 10 March 2023
Revised : 24 April 2023
Accepted : 27 April 2023

Keywords:
Gummy jellies, Wheatgrass, Antioxidant activity, Physicochemical properties, Texture properties, Nutritional properties

Abstract

The aim of this study was to develop gummy jellies from wheatgrass leaf juice, assess the antioxidant activity of wheatgrass, and study the effects of gelatin (10, 20, 30 and 40% of honey weight) on the physicochemical properties and texture using different techniques (warm and hot), as well as evaluate the nutritional property of selected formulation. The antioxidant activity of wheatgrass was determined using the DPPH assay. The 50% effective concentration (EC_{50}) was 23.42±1.13 mg sample/mL. This concentration was then used in formulation of gummy jellies. The samples were investigated for moisture, water activity, pH, color, and texture. The moisture, water activity and pH were in the range of 0.66±0.07 - 6.20±0.62 %, 0.72±0.03 - 0.87±0.02 and 4.76±0.08 - 5.92±0.04, respectively. The result suggested that gummy jellies had low moisture, water activity and pH, particularly the 10% honey weight of gelatin concentration using the hot technique (H1 formulation). These low values contribute to potentially increase in their shelf life. The gummy jellies were red-yellowish in color. The texture of the H1 formulation was not significantly different ($p>0.05$) in hardness, gumminess and chewiness compared to 20-30% honey weight of gelatin using both hot and warm techniques. Therefore, the H1 formulation was selected for the analysis of nutritional properties. The energy of the H1 formulation was 56 kcal/50 g per one serving. Most of the energy of the gummy jelly came from sugar (18 g). According to the recommendation by WHO, adults and children should consume less than 25 grams of sugar per day to reduce the risk of NCDs. Therefore, the gummy jelly is suitable for less than 2 servings per day.

Introduction

Gummy jelly is a confectionery product in a group of candy gel. Commercial gummy jellies are products based on sugar, gelling agent and food additives (Cedeno-Pinos et al., 2021). The commonly used gelling agents are gelatin, pectin and starch (Takeungwongtrakul et al., 2020). Gelatin is used as a main gelling agent that provides structure in the gummy jellies (Mutlu et al., 2018). The popular sweetener sources are glucose,

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sucrose, syrups which have a high glycemic index and contribute to high blood sugar level (Jiamjariyatam, 2018). Long-term high blood glucose levels may induce obesity, cardiovascular disease, diabetes and other diseases, which are all serious threats to human health (Gan et al., 2022). Reducing or replacing sugars with honey might represent healthier alternatives. Honey is a functional food produced by bees (*Apis mellifera*) which contains a wide variety of bioactive substances and enzymes giving it antioxidant, antimicrobial, anti-inflammatory and anticarcinogenic properties (Mutlu et al., 2018). Replacing sucrose with honey also decreases the high glycemic index of the product (Rivero et al., 2020). Food additives of gummy jelly product also have negative effects on health. In the past years, several fruit, vegetable and herb have been considered for the development of functional gummy jellies (Teixeira-Lemos et al., 2021; Takeungwongtrakul et al., 2020; Charoen et al., 2015) such as gummy jelly mixed with *Thunbergia laurifolia* Linn. extract aimed to reducing breath alcohol content (Kitpot et al., 2020). Therefore, an addition of healthier ingredients in gummy jelly may be less detrimental to health by the product.

Wheatgrass (*Triticum aestivum* Linn.) belongs to the family Poaceae. The juice of wheatgrass is known as "green blood" because it contains a large amount of chlorophyll, making up 70% of the total chemical constituents (Choudhary et al., 2021). Chlorophyll was shown to have positive effects on thalassemia patients undergoing blood transfusion and reduce toxicity in cancer patients (Ove et al., 2021). Wheatgrass juice is also an abundant source of essential vitamins and minerals such as retinol, ascorbic acid, tocopherol, vitamin B complex, calcium, phosphorus, magnesium, alkaline earth metals, potassium, zinc, boron and molybdenum (Adhikari et al., 2022). Clinically, wheatgrass juice is mainly utilized because of its antioxidant property. It is reported that wheatgrass contain antioxidant compounds such as carotenoids, tocopherols, tocotrienols, phenolic acids, phytic acid, phytosterols and flavonoids (Kaur et al., 2021). Furthermore, it is a significant herbal plant used to treat various diseases and disorders such as high blood pressure, obesity, cancer, diabetes, kidney swelling, gastritis, ulcers, astriction, anemia, eczema, pancreas, diuresis, fatigue, anemia, asthma, hemorrhoids, halitosis, skin problem and constipation (Choudhary et al., 2021; Jiang et al., 2021).

This study aimed to develop gummy jellies from wheatgrass juice containing antioxidants and investigate effects of gelatin content (10, 20, 30 and 40% of honey weight) on the physicochemical properties and texture using different techniques (warm and hot) as well as evaluate nutritional properties of selected formulation. The results would be useful as a guide to further develop commercial products that are also healthier alternatives.

Materials and methods

1. Preparation of wheatgrass leaf juice

Fresh wheatgrass leaves were obtained locally from Namai, Ladlumkaew, Pathumthani Province, Thailand. Wheatgrass leaves (60 g) were cleaned before squeezing with a juicer MC-911-1 (Jyu Fong Machinery Co., LTD, Taichung, Taiwan). The sample was centrifuged at 4500 rpm for 10 min. The percent extract (% yield) was calculated using the formula below (Smith et al., 2015). Then, the antioxidant activity of the extract was measured and applied on healthy gummy jelly supplement.

$$\%\text{yield} = \frac{wheat\ grass\ juice}{initial\ weight\ of\ wheat\ grass\ leaves}\ x\ 100$$

2. The 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) free radical scavenging assay of wheat grass leaf juice

The antioxidant activity method was adapted from Sembiring et al. (Sembiring et al., 2018). Different concentrations of standard (ascorbic acid, 1-10 µg/mL) were prepared. One-hundred microliter of either standard (Sigma-Aldrich, St Louis, MO, U.S.A.) or sample (200 - 1000 mg sample/mL) solutions were mixed with 100 µL of 208 µM DPPH (Sigma-Aldrich, St Louis, MO, U.S.A.) in methanol (Merck, Darmstadt, Germany). After incubating in darkness for 30 min, the absorbance was measured at 517 nm using a microplate reader. All reactions were carried out in triplicate. Antioxidant capacity was expressed as the concentration of extract providing half maximal effective concentration (EC_{50}). The EC_{50} was determined graphically by plotting the percent inhibitions (%inhibition) against the extract concentrations. The percent inhibition was calculated using the following formula:

$$\%inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where: $A_{control}$ is the absorbance of the DPPH solution
A_{sample} is the absorbance of sample or standard with DPPH solution

3. Preparation of healthy gummy jelly

Gummy jelly was prepared using two techniques (Mutlu et al., 2018), warm (50-55°C) and hot (115°C) with four different gelatin doses (10, 20, 30 and 40% of honey weight). The formulations are shown in Table 1. Each gelatin (McGarett, JR F & B Co., Ltd., Bangkok, Thailand) dose was added to 100 mL of distilled water and kept for 5 min. Then, excess water was completely removed and the soaked gelatin was used as gelling agent. The soaked gelatin was dissolved at 70°C for 30 min in a water bath (Memmert, Schwabach, Germany). In the warm technique, dissolved gelatin was mixed with 50 g of honey (the Royal Chitralada Project, Bangkok, Thailand) and 23.42 mg/mL of wheatgrass juice. During mixing at 200 rpm for 1 min with a stirrer (Daihan Scientific, Gangwon-do, Korea), the mixtures were kept at 50-55°C. For the hot technique, the soaked gelatin was added into the 50 g of honey and 23.42 mg/mL of wheatgrass juice. The mixtures were manually stirred by a glass rod on a hot plate for 20 min, to ensure that the gelatin did not set before reaching the desired temperature. During this process, the temperature of mixture was kept at approximately 115°C. Mixtures from both techniques were poured into a bear shape silicone mold (width x length x thickness; 10 x 10 x 5 mm) and kept for 30 min in a fridge at 4°C. The jelly gummies were removed from the mold and kept for three days in a desiccator. After this process, the honey jelly gummies were placed in plastic bags and stored at 4°C.

Table 1 Formulations of healthy gummy jelly from wheatgrass juice

Formulations	Gelatin doses (g) (% of honey weight)	Wheatgrass juice (mL)	Honey (g)
Warm technique			
W1	5 (10)	0.64	50
W2	10 (20)	0.70	50
W3	15 (30)	0.77	50
W4	20 (40)	0.82	50
Hot technique			
H1	5 (10)	0.64	50
H2	10 (20)	0.70	50
H3	15 (30)	0.77	50
H4	20 (40)	0.82	50

4. Physicochemical analysis

The moisture content measurement was performed using a moisture analyzer MA37 (Sartorius, Goettingen, Germany). The condition was set to the fully automatic mode. Ten grams of gummy jelly sample was approximately weighed on the moisture analyzer. Water activity was measured by the auto start mode using water activity meter LabSwift-aw (Novasina, Lachen, Switzerland). The whole gummy jelly sample was added to a sample dish then put into the water activity meter. After the analysis, the actual values in water activity and moisture were recorded as a_w and % moisture, respectively. The measurements were carried out in triplicate.

The pH value was determined by homogenizing the sample (1 g) in distilled water (9 mL) at 50°C and measured using a digital pH meter (SI analytics, Mainz, Germany). The measurements were carried out in triplicate.

The color of gummy jelly was analyzed using Chroma Meter CR-400 colorimeter (Konica Minolta, Tokyo, Japan). The color meter was calibrated using a white plate CR-A43 and assessed using the DP mode. The measurement was carried out in triplicate. The analyzed color parameters were L*, a*, b* using the following implication: L* from (0) black to (100) white, a* from (-)greenness to (+) redness, b* from (-) blueness to (+) yellowness.

Texture analysis of gummy jelly was evaluated using texture analyzer CT3 (Ametek Brookfield, Middleborough, MA, U.S.A.) equipped with the cylindrical (35 mm.) probe with texture profile analysis (TPA) mode using the test speed of 1 mm/s. The hardness, adhesiveness, cohesiveness, springiness, gumminess and chewiness were reported. The measurement was carried out in triplicate.

5. Nutritional analysis

The selected sample were analyzed for nutritional values (total energy, carbohydrate, protein and fat) using the nutritional analysis program (INMUCAL-Nutrients version 4.0, Institute of Nutrition, Mahidol University). The obtained data were reported as 50 g of gummy jelly sample (1 serving).

6. Data analysis

The experiment was performed using a completely randomized design. Statistical data were analyzed for variance using ANOVA (SPSS software, version 29). The LSD's test was applied to detect the differences among the samples. The mean difference is significant at the 0.05 level ($p \leq 0.05$).

Results and discussion

1. Antioxidant activity of wheatgrass leaf juice

The fresh wheatgrass leaves were processed by squeezing and gave the final yield of approximately 50 w/v. The antioxidant activity of wheatgrass juice at 50% effective concentration (EC_{50}) was 23.42±1.13 mg sample/mL. This concentration of the wheatgrass juice resulted in a 50% free radical (DPPH) inhibition. There are reports that free radical is abundant in the cell of the human body and can cause biological damage (Martemucci et al., 2022). Therefore, the 23 mg/mL of sample juice was added to gummy jelly.

2. Physicochemical properties

The moisture and water activity were in the range of 0.66±0.07-6.20±0.62 % and 0.72±0.03-0.87±0.02, respectively (Table 2). The increase in the moisture content and water activity of gummy jellies may be affected by the mixing technique and gelatin dose, as in formulations with higher gelatin content may not have been vaporized enough during the hot mixing. The moisture content of gummy jellies was lower than the general recommendation (less than 20%) for this type of product (Renaldi et al., 2022). Moreover, the moisture content of W1 (0.66±0.07) formulation was low and not significantly different ($p>0.05$) from the H1 (0.77±0.25) and H2 (1.30±0.65) formulations. Probably due to the gelatin content was not quite different, resulting in the moisture content of 3 formulations were not different. The water activity affects the growth of microorganisms which negatively correlates with the stability of the products. It is reported that the water activity in the range of 0.7 – 0.8 of a gummy jelly had a high stability (Periche et al., 2014). In particular, the W1 and H1 formulations had no significant difference in low water activity. Therefore, the low moisture content and water activity of W1 and H1 may enable good conservation.

The pH values of the gummy jellies are shown in Table 2. The pH values of gummy jellies ranged from 4.76±0.08-5.92±0.04. The pH values of gummy jellies were affected by the mixing technique and gelatin dose. The pH of the hot mixing technique decreased because the hexose was oxidized to acidic sugar at high temperature (Mutlu et al., 2018). The result showed that the increase in gelatin resulted in an increase of the pH value. This is because the gelatin was derived from collagen which is an amino acid and is known to have high pH value of gummy jellies (Jiamjariyatam, 2018). Periche et al. (2014) reported that less of pH could affect the increase in their shelf life. Specifically, the pH value of H2 formulation showed the lowest value but was not significantly different from that of the H1 and W1 formulas. The results suggested that H1, H2 and W1 formulations might have a longer shelf life compared to other formulations.

The L*, a* and b* values of the gummy jellies ranged from 27.23±0.67-32.12±1.74, 0.24±0.03-1.21±0.05 and 1.92±1.65-7.10±0.63, respectively (Table 2). The high gelatin content tends to increase brightness. The positive a* and b* values showed that the gummy jellies were red-yellowish in color. This result correlated with a study of Rivero et al. (2020) with a positive a* and b* value of the honey gummy jelly (Rivero et al., 2020). The yellow pigment of gummy jellies was probably derived from the phytochemical compounds in the honey such as rutin, caffeic acid, quercetin and kaempferol (Vazquez et al., 2021). These compounds were major groups of phenolic acid and flavonoids which are responsible for the antioxidant activity of honey (Hossen et al., 2017; Rubio-Arraez et al., 2016). The highest a* value was of the H1 (1.21±0.05) formulation. The b* value of W4 (7.10±0.63) formulation was the highest, followed by H1 (5.58±0.26) and H3 (5.18±0.26). The results indicated that H1 formulation was high in all a* and b* values.

Table 2 Moisture, water activity, pH, and color of gummy jellies

Formulas	properties					
	Moisture (%)	Water activity (a_w)	pH	Color		
				L*	a*	b*
W1	0.66±0.07^{e}	0.72±0.03^{c}	5.00±0.05^{c}	27.23±0.6^{7c}	0.30±0.08^{cd}	2.68±0.61^{d}
W2	2.03±0.37^{d}	0.79±0.01^{b}	5.70±0.07^{a}	32.12±1.74^{a}	0.68±0.83^{b}	3.58±0.15^{cd}
W3	2.84±0.27^{c}	0.85±0.05^{ab}	5.91±0.09^{a}	27.67±0.91^{c}	0.41±0.14^{cd}	2.72±0.30^{d}
W4	4.98±0.11^{b}	0.87±0.02^{a}	5.92±0.04^{a}	30.26±0.19^{b}	1.04±0.17^{a}	7.10±0.63^{a}
H1	0.77±0.25^{e}	0.76±0.04^{bc}	4.77±0.15^{cd}	29.86±0.32^{b}	1.21±0.05^{a}	5.58±0.26^{b}
H2	1.30±0.65^{e}	0.81±0.05^{b}	4.76±0.08^{cd}	30.90±0.04^{ab}	0.50±0.23^{bc}	2.55±0.44^{d}
H3	6.20±0.62^{a}	0.85±0.03^{a}	5.58±0.54^{ab}	31.19±0.15^{ab}	1.20±0.14^{a}	5.18±0.26^{b}
H4	4.80±0.52^{b}	0.86±0.04^{a}	5.22±0.19^{bc}	30.69±0.78^{b}	0.24±0.03^{d}	1.92±1.65^{de}

Remark: $^{a\text{-}c}$ different letters in the same column indicate values that are significantly different in statistics ($p\leq0.05$).

3. Texture properties

The texture profiles of gummy jellies including hardness (233.50±7.78-1020.50±44.55 g), adhesiveness (0.01±0.00-0.10±0.14 mJ), cohesiveness (0.82±0.32-0.95±0.16), springiness (4.66±0.15-4.80±0.15), gumminess (181.50±44.55-924.50±10.61 g) and chewiness (8.30±2.26-42.75±2.92 mJ) are shown in Table 3. Different gelatin contents and techniques had no significant impact on springiness, cohesiveness as well as adhesiveness properties ($p>0.05$). However, the texture indicators that are particularly relevant to gelled confections are hardness, chewiness and gumminess, because these indicators affect consumer preferences. The hot technique resulted in a stronger gummy jelly than the warm technique. This may be due to stronger intermolecular forces when the mixture was combined at high temperature. Adding 10-40% of honey weight of gelatin in all warm and hot (except for H4) techniques resulted in a decrease in hardness. A study showed that acid hydrolysis of gelatin contributed to the decrease in the strength of gelatin (Charoen et al., 2015). In this research, it may be due to the addition of more wheatgrass, which is reported to have an acidic pH of 5 (Rodriguez et al., 2022). The H4 formulation had the highest hardness possibly due to the gelatin content and high temperature applied, which led to higher level of gel cross-linking and network formation (Rebers et al., 2019). The chewiness and gumminess also showed the same trend as hardness. The results indicated that the concentration of 10% honey weight of gelatin using hot technique (H1) did not affect the hardness, gumminess and chewiness compared to 20-30% honey weight of gelatin using both hot and warm techniques. Based on the above information, H1 has suitable moisture, water, pH and color. Therefore, H1 is selected to study nutritional properties.

Table 3 The texture profile of gummy jellies

Technique	properties					
	Hardness (g)	Adhesiveness^{ns} (mJ)	Cohesiveness^{ns}	Springiness^{ns} (mm)	Gumminess (g)	Chewiness (mJ)
W1	313.50±4.95^{b}	0.10±0.00	0.84±0.03	4.68±0.02	264.00±4.24^{b}	12.10±0.28^{b}
W2	288.00±1.41^{b}	0.10±0.14	0.87±0.06	4.71±0.0 2	248.50±16.26^{b}	11.50±0.71^{b}
W3	270.50±6.36^{b}	0.05±0.07	0.95±0.16	4.80±0.15	258.00±48.08^{b}	12.15±2.62^{b}
W4	233.50±7.78^{c}	0.40±0.57	0.82±0.32	4.66±0.15	181.50±44.55^{c}	8.30±2.26^{c}
H1	581.00±57.98^{b}	0.05±0.07	0.93±0.00	4.80±0.03	540.00±53.74^{b}	25.40±2.40^{b}
H2	449.00±0.00^{b}	0.01±0.00	0.90±0.01	4.77±0.11	405.00±5.66^{b}	18.95±0.78^{b}
H3	343.50±55.86^{b}	0.10±0.00	0.92±0.07	4.75±0.05	315.00±26.87^{b}	14.65±1.06^{b}
H4	1020.50±44.55^{a}	0.45±0.49	0.91±0.05	4.72±0.16	924.50±10.61^{a}	42.75±2.92^{a}

Remark: $^{a\text{-}c}$ different letters in the same column indicate values that are significantly different in statistics ($p\leq0.05$), ^{ns} is not significantly different in statistics ($p>0.05$) in the same column.

4. Nutritional properties

The nutritional properties of the H1 formulation were analyzed as shown in Table 4. Total energy of the H1 formulation was 56 kcal/50 g. Most of the energy of the gummy jelly was attributed to carbohydrate, which was 14 g and comprised of glucose (6 g) and fructose (8 g). The developed formulation showed a lower energy when compared to the commercially available formulations, reported by Teixeira-Lemos et al. at 175.5 kcal/50 g (Teixeira-Lemos et al., 2021). The world health organization (WHO) guideline recommended that adults and children should consume less than 25 grams of sugar per day (Huang et al., 2023). The guideline provides additional health benefits to reduce the risk of NCDs (WHO, 2015). Therefore, the gummy jelly was suitable for less than 2 servings/day.

Table 4 Nutritional properties of H1 gummy jelly

Nutritional properties	Nutritional values
Total energy, kcal/50 g	56
Carbohydrate, g (% of total energy)	14 (100)
Sugar, g	14
- Glucose, g	6
- Fructose, g	8
Protein, g (% of total energy)	0 (0)
Fat, g (% of total energy)	0 (0)

Conclusion

Gummy jellies were made from different gelatin concentrations using hot and warm techniques. The formulation used wheatgrass juice in the 50% effective concentration and replacing sugar with honey. Gummy jellies exhibited low water activity, moisture and pH, especially the H1 formulation. The reddish yellow of gummy jellies were probably derived from the pigments found in honey and wheatgrass that are beneficial to consumers. The hot technique resulted in a stronger gummy jelly than the warm technique. The gumminess was not different between the H1 formulation and all

other formulations, except for H4. The nutritional properties of the H1 formulation suggested that the gummy jelly was suitable for less than 2 servings/day. According to the above results, the H1 formulation (10% honey weight of gelatin with hot technique) could be recommended for a gummy jelly manufacturing process.

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Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Instructional Design: Under the Concept of Game Based Learning for Nursing Education

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Article info

Article history:
Received : 4 March 2023
Revised : 21 April 2023
Accepted : 28 April 2023

Keywords:
Game based learning,
Instructional design

Abstract

Game-based learning (GBL) is a method that motivates students to learn content in a fun way. Learners are motivated by challenge, fantasy, and curiosity in games. GBL provides learners with the opportunity to test the game and empower them to make decisions. The course content can be inserted into the minds of the learners through the game to help them develop and achieve higher learning outcomes. GBL can be used for both traditional classroom teaching and online teaching. This article presents information about game-based learning management. The concept of game-based learning and a review of the design elements of educational games are discussed. We then review the game development process and the implement of games in the classroom as well as assessing outcomes. Lastly, GBL trend for nursing education is discussed.

Introduction

Higher education is essential to society because it prepares graduates to work or train for a career. Learning by memorizing or studying by lecture is not enough in this era of high competition. It is necessary that a change in learning style must be implemented through proactive learning management. Instructional design should be changed from a lecture to learning activities and involve actively engaging students in teaching and learning activities in the classroom. Teachers become the director of learning management through learning activities (Chooha et al., 2019). When the role of the teacher become a facilitator of knowledge, then it is possible to design a classroom for effective learning and implement teaching and learning activities effectively. Student's understanding arises when the teacher offers opportunities for learners to create knowledge for themselves.

The global Covid-19 pandemic has led to an urgent change in the education of nursing students from traditional to distance learning. The active engagement of nursing students has deteriorated over time. Therefore, there has been a widespread search for teaching methods that balance learning, enjoyment and active student participation, such as game-based learning (GBL). (Tavares, 2022). Game-based learning (GBL) is a new teaching tool in the field of education research. Researchers have highlighted various benefits of using GBL, such as improved academic performance (Hwang & Chang, 2020), higher learning motivation, and greater retention of acquired knowledge (Kao, 2020). The result of research on applying game-based learning to enhance the participation of students proved that adding game elements in operating system courses can increase the level of attraction of syllabus to students and the students would be willing to spend time in

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courses because they want to win, thus fulfilling the purpose of elevating learning motivation (Lai et al., 2012). In addition, Camacho-Sánchez et al. (2023) identify GBL and gamification as significant learning methods due to their impact on student motivation, academic performance and commitment to improving health and physical performance.

To meet the developing needs of today's nursing students and the demand for 21st-century skills in current healthcare, game-based learning is increasingly becoming a strategy to complement simulation strategies in nursing education. It can improve learning outcomes while enhancing learning experiences by promoting engagement and motivation (Xu et al., 2021). At present, nursing education is rapidly evolving. Game-based learning has been gradually used for education and several innovations have emerged. The emergence of serious games and gamification provides alternative approaches for educators to improve the nursing teaching process. Both serious games and gamification exert their education-promoting function by providing the possibility of combining learning activities such as feedback, testing and spaced repetition with active participation and autonomy as well as positive experiences for students. Developing effective GBL modalities has the potential to bring immersive experiences for medical students and improve their study outcomes. Herein, we reviewed recent studies employing GBL in medical education, including serious games and gamification teaching. Furthermore, we also discussed the effectiveness and limitations of GBL to suggest future directions for the development and application of GBL in nursing education.

Game-based learning activities can create a fascinating learning environment for students to improve their study outcomes. The benefits of GBL on learners include enhancing their collaborative awareness, providing them with opportunities for active learning to better solve clinical problems and improving their clinical reasoning and decision-making skills. Furthermore, GBL can enable educators to explore novel and feasible teaching strategies, which contribute to the reformation of current didactical activities. Therefore, the application of serious games and gamification in medical education is meaningful (Jan & Gaydos, 2016; Gudadappanavar et al., 2021; Xu et al., 2023).

This article presents information about game-based learning management. The concept of game-based learning and a review of the design elements of games. We then review the game development process and then discuss the use of games in the classroom and assessment. Lastly, GBL trend for nursing education is discussed.

The concept of game-based learning

1. Definition of game-based learning

Kirriemuir & Mcfarlane (2004) described game-based learning (GBL) as a medium of gaining knowledge and skill acquisition via gameplay where game activities require the player to solve problems and challenges provided to gain achievement. GBL is the borrowing of certain gaming principles and applying them to real-life settings to engage users. The motivational psychology involved in GBL allows students to engage with educational materials in a playful and dynamic way. GBL is not just creating games for students to play, it is designing learning activities that can incrementally introduce concepts and guide users toward an end goal (Pho & Dinscore, 2015). GBL describes an environment where game content and gameplay enhance knowledge and skills acquisition and where game activities involve problem-solving spaces and challenges that provide players/learners with a sense of achievement (Qian & Clark, 2016). GBL is a style of learning that uses games as an instructional method to promote effective learning. A game helps attract learners' attention and allows students to have fun while learning (Jan & Gaydos, 2016).

Summary, GBL is where game characteristics and principles are embedded in learning activities that promote student engagement and learning motivation. GBL is also an active learning technique where games enhance student learning with a learning context designed by teachers. Components of GBL include point systems, discussion boards, quizzes and classroom response systems.

2. Types of game-based learning

Exploring the world of GBL will open the door to many types and examples of games. Compared to games outside of the education space, "serious games" are ones designed to teach or help students practice specific skills or content. Dimitra et al. (2020) described some of the most common type of game-based learning that include:

2.1 Flash Cards are games that uses a traditional or game-specific deck of cards. "War" is a traditional card game that can have a mathematical twist. The study of Fernandes et al., (2021) identified the use of card games with patients in palliative care, assessed self-reported

satisfaction and synthesized findings on the effectiveness of its application. The card game to facilitate conversations with patients in palliative care is a productive and effective approach to discussing uncomfortable topics of death, dying and end-of-life care.

2.2 Board games include playing on a board that usually involves the movement of pieces. Chess and checkers are popular ones, but there are hundreds if not thousands of board game to explore. A gerotechnology board game was implemented in clinical nursing practices and functioned as a playful resource to exercise self-determination and independence among older adults, boosting memory, self-esteem, socialization processes, exchanging experiences and shared learning (Olympio & Alvim, 2018). In Thailand, board games are used to teach financial literacy among teenagers and improves the critical thinking skill of people (The Nation Thailand, 2018).

2.3 Simulation games are designed to closely simulate real-world activities. For example, Koivisto & Eriksson (2020) designed a gamified simulation to evaluate the effectiveness of a simulation game on nursing students clinical reasoning skills.

2.4 Quiz games are typically designed to explore the properties of language or the ability to use a language itself such as Kahoot game. In prior research the effects of Kahoot game-based versus nongame-based learning achievements and anxiety among nursing students showed that: Kahoot improves learning achievements and decreases agitation among students in game-based groups compared to nongame-based groups (Ofori et al., 2020).

2.5 Puzzle games emphasizes puzzle solving through one's use of things such as logic, word completion, sequence solving, as well as spatial and pattern recognition.

2.6 Reality testing games are electronic games wherein players can manipulate what appears on the screen such as augmented reality (AR) and virtual reality (VR). The virtual simulation was found to have high-quality outcomes that are related to the student experience and satisfaction with the learning environment, acquisition and retention of knowledge correlated to clinical concepts and the development of performance-based skills, including performing procedures with improvements in the ability to complete the scheme and efficiency during the step (Lee et al., 2020).

2.7 Role-playing games are interactive which players assume the role of imaginary characters who engage in adventures.

3. The benefits of game-based learning

Game-based learning can create a dynamic that can inspire learners to develop skills and competencies as they focus on the activities of the game. They can function as individual learning activities, a powerful content delivery mechanism over several sessions and last for the duration of the course. To deliver a range of a game, faculty members tend to divide the syllabus into levels through which the students must progress, with students getting feedback rather than grades. For it to be effective, the game must align with learning outcomes and should not be competitive in the conventional sense. The benefits of game-based learning are as follows: 1) helps in retaining learning insights, 2) learning through familiar devices, 3) stimulating imagination, 4) learning to last a lifetime, 5) helps in simplifying complex concepts, 6) learning by doing, 7) improving sales, 8) collaborating, 9) reducing chances of failure in the real world and 10) higher receptivity to learning (Hurix, 2023). A well-designed game and supporting materials in the classroom can make education more relevant by allowing students to assume different roles, confront problems, make meaningful choices and investigate the consequences of these choices. Teachers are now confronted with larger classrooms with widely disparate capabilities. These types of learning materials and educational board games allow students to take on various challenges, fail in a safe environment and eventually succeed and level up at their own pace. The use of games in the classroom allows students to develop an emotional connection to learning and subject matter. It gives them the chance to get feedback and practice. The games can also be tailored to fit specific teaching situations. Furthermore, games encourage students to participate more actively in their classes and healthy competition can boost their motivation. As students feel more capable of achieving their interim goals, they feel more successful and can see their progress. They also learn about the availability and value of alternative procedures for gaining a better understanding of their subjects. All of this helps students become more confident, independent thinkers who are better prepared to take on large projects and see them through to completion. While we have accepted that games in class are beneficial in key aspects of a student's learning, let us now look at how games work to improve multiple aspects of a student's learning.

The game development process

Game-based learning is a great way to improve student learners' creativity, critical thinking, and problem-solving skills. It is based on the use of imagination. Instructors can give students the freedom to come up with solutions and ideas which boost their level of creativity. Game-based learning incorporates game characteristics and principles into learning activities. Learning activities inspire student engagement and enthusiasm to learn. The motivational psychology involved in game-based learning allows students to engage with educational materials in a playful and dynamic way. Points systems, badges, leaderboards, discussion boards, quizzes and classroom response systems are all components of game-based learning (Prodigy, 2021; Bulut et al., 2022).

From our lesson to learn, we concluded that the principles of game-based learning consist of 1) Practice game-based learning design must cover various exercises. 2) Learning by doing must focus on a self-learning, which self-learning will allow learners to understand more deeply. 3) Learning from mistakes, allowing learners to learn from mistakes. It does not hurt to learn from mistakes. 4) The game must have a clear goal for goal-oriented learning. What to let students learn so that the learners try to achieve their goals and 5) The learning point must contain all the relevant information or critical issues that the learners should learn so that students can apply that knowledge to practical use.

Games may help us obtain and remember facts and figures, but games are best utilized when designed around a concept as a learning goal. Therefore, we use the ADDIE Model process in this context to create game-based learning (Aldoobie, 2015; Prachuaplap, 2016). The ADDIE model is an instructional design model used by training developers and other educators. It consists of five critical phases: Analysis, Design, Development, Implement and Evaluation.

1. Analysis

1.1 In the analysis phase, instructional problem is clarified, the instructional goals and objectives are established, and the learning environment and learner's existing knowledge and skills are identified. Below are some of the questions that are addressed during the analysis phase:

* Who is the audience and their characteristics?
* Identify the new behavioral outcome?
* What types of learning constraints exist?
* What are the delivery options?

1.2 Analyze relevant factors such as the number of students' basic knowledge and learner's learning styles. Analyze the content to determine the scope of the content. Prioritize content to connect relationships by defining main topics, subtopics and resources conducive to development to formulate a development plan.

2. Design: Designing the game

2.1 Write down your ideas. You never know when the perfect inspiration is going to hit. You may find that combining two different ideas makes a neat new game concept. Keep a log of ideas in a notebook, on your computer, or in a note-taking app on your phone.

2.2 Develop your game with a theme. Themes are the "feel" of a game and can also be referred to as the types of game-based learning.

2.3 Use mechanics to develop your game, alternatively. Mechanics are the ways players interact with the game and each other. In Monopoly, the mechanics are centered around dice-rolling, buying/selling property and making money. The mechanics of Axis & Allies involve moving pieces across a large board and resolving player conflicts with dice rolls.

2.4 Determine the age range of your players. The age range of your players will influence the complexity of your game board and its rules. If you are designing a game for students, your game should be simple, easy to understand and fun.

2.5 Set player, time and size limits for your game.

2.6 Decide how players will win. Once you have the basic ideas behind your game written down, ask yourself, "What are the winning conditions of this game?" Consider the different ways the player could win and keep these in mind as you work on the game.

2.7 Write out the basic rules. These will undoubtedly change as you continue to develop your game, but a basic set of rules will allow you to begin testing quickly.

3. Development: Making a prototype and playtesting the prototype

Make a prototype to use and evaluate your game (Alpha testing to game designers). This is a phase of the process after game design elements have been brainstormed and the creation of a playable game is started. Game designers can use anything in creating a prototype. Before you begin work on the finished product, create a rough prototype so that you can play around with it. It does not have to be pretty, but a hands-on experience will help you to see if the basics work the way you planned. A prototype is a vital part of

the game creation process, as it gets ideas out of your head and into the real world where you can evaluate them with other players. Hold off on adding artistic details until you begin assembling the final product.

3.1 Sketch a rough draft of your board design. In this step, you will use your storyboards as a guide in creating your game to ensure that you are on the right track.

3.2 Assemble prototype game pieces. Buttons, checkers, poker chips, chess pieces and knickknacks work well as prototype game pieces. Avoid using game pieces that are too large for your prototype, since these can make it difficult to read information written on the board. Game pieces can change considerably over the course of your game's development. Keep prototype game pieces simple so you do not invest a lot of time designing something that ends up getting changed.

3.3 Use game cards to add variation. Randomly shuffled game cards will affect players in unexpected ways. A card often tells a quick story about an event that befalls a player and then changes their score/position/inventory accordingly.

If prototyping is the alpha test, playtesting is the beta. This phase can be the most frustrating, but also the most important part of the design process. It is important that you find people to play the game who are not biased to the designers and who are not told how to play the game. Observing gameplay will allow designers to revise game elements.

3.4 Test your prototype by yourself. Once you have all the basics assembled for your prototype, you can start testing the game to see how it plays. Before trying it out on a group, play it by yourself. Play through the game as each player and record any positives or negatives you notice as you play.

3.5 Test your game with friends. After you solo played your game enough to work out most of the kinks, it is time to playtest. Gather some friends and explain that you would like them to test your game. Let them know that it is a work in progress and that you'd appreciate any feedback.

3.6 Switch up the test players for a better perspective of your game. Everyone approaches games differently and some might see things missing that you would not have realized on your own. The more people you get to test your game, the more opportunities you will have to find flaws or weak points and fix them. Refine your prototype throughout testing. As you finish each playtest, make necessary changes or adjustments to your game board, rules and other components. As you continue to test, keep track of the features that you have changed.

4. Creating the Final Product

4.1 Make a list of needed materials. Once testing is complete and you are happy with your game, you can get started on the final version. Each game will have unique needs, so your materials may vary. Make a list of all the parts your finished game will require so you do not forget anything.

4.2 Illustrate your board. Your game board is the centerpiece of your board game, so feel free to get creative with the design. Make sure that the path or playing field is clearly marked and that all the instructions on the board are easy to read.

4.3 Create the game pieces. The simplest way of doing this is by drawing or printing images on paper and then taping or gluing them to a sturdy backing, like cardstock. If you are making a game for family or friends, you can even use real photos of players.

5. Implement: Implement GBL in the classroom

This phase is about transforming the plan into action. Instructors proved the influence of game-based learning strategies on students' learning effectiveness. In particular, the combination of game-based activities and flipped teaching distinguished from traditional flipped instruction, which focuses on video watching or using static digital teaching materials. Thus, this study developed a digital game based on the content of the learning units in the teaching materials. Students arranged to play the game before engaging in classroom learning activities in the flipped classroom. For instance, to investigate its effectiveness in boosting learners' motivation, this study conducted an experiment where the experimental and control groups engaged in different learning activities.

6. Evaluation

Their learning effectiveness was then assessed and the participants of the experimental group filled out a learning perception questionnaire at the end of the game-based learning activities. The results were analyzed and discussed. The evaluation phase consists of two parts: formative and summative. Formative evaluation is present in each stage of the ADDIE process. Summative evaluation consists of tests designed for domain specific criterion-related referenced items and providing opportunities for feedback from the users.

In this part, the author summarizes the design of a measurement and evaluation tool based on learning objectives from using board games. Measurement and evaluation tools include tests, interview forms,

questionnaires, measurement forms and lessons learned, which are analyzed to be used to measure and evaluate using board games for learning as follows:

6.1 Knowledge, memory, comprehension, classification and conceptual content should use lessons learned and quizzes to focus mainly on the development of student achievement. The advantage is that knowledge is precise. The disadvantage is that measuring and making measurements separate from learning activities is too stressful.

6.2 The content is related to the rules and their implementation. Lessons learned, questionnaires, interview forms, or subjective tests should be used. Because the nature of the commission, students can design their performance based on their opinions.

6.3 Game-based learning is widely employed and, if successfully executed, significantly enhances students' engagement and skill in learning in the classroom. It is essential to measure skill improvement to stay informed of the students' progress. Therefore, an assessment checklist and observation should be used by an instructor to measure and evaluate a student's skills.

6.4 Attitude-related content: educators used lessons learned, questionnaires and interview forms because the desired outcome is partial based on the way students think and feel about game playing.

Under the measurement and evaluation approach, there are tools to seek answers to the outcomes of using learning games. Measuring and evaluating lead to the learning outcome should not use the game once or once and then measure the result because it cannot lead to the development of students. Students learning development takes a reasonable amount of time with both formative and summative evaluation in the classroom. The focus is on inter-process evaluation, which uses single-point rubric assessment criteria. In addition, each person is sensitive to thinking and making decisions while playing the board game. Therefore, the use of simple measures must be able to capture student behavior. It may be an expression of gestures, words, and conversations in the game, which can be considered and easier evaluated with rubrics.

Game-based learning was used for many different courses or content in nursing education. Simulation games were the most used game type. Game-based knowledge facilitated the achievement of learning outcomes primarily in the cognitive domain. Some gamification and design-related aspects of game-based environments were evaluated as positive and negative. Game-based education helps assess learning outcomes in only three studies.

The use of game-based education intervention was found to have enhanced the nursing students' learning experiences, given its advantages as being motivating, engaging, enjoyable and conductive. Game-based learning is an alternative educational approach that can support and complement current teaching methods in nursing (Xu et al., 2021). Even with the emerging technologies in game-based learning, board games will still be relevant in many situations. Due to the divergence of media and technologies, board games are being re-introduced on tablets and online.

We summarized the process of game instructional design for evaluation as shown in the Fig. 1.

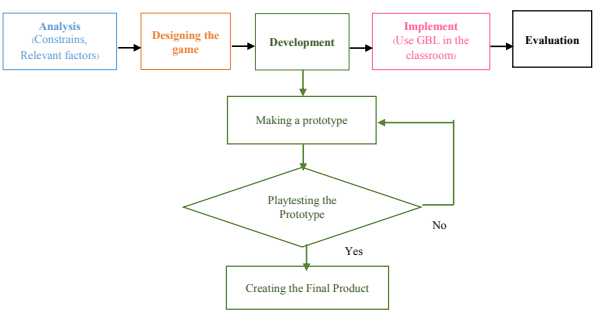

Fig. 1 Process of game instructional design for evaluation

Evidence of impact and outcomes of game-based learning

Previous reviews indicate that the most frequent outcome-investigated in educational game studies was knowledge acquisition (Connolly et al., 2012; Li & Tsai, 2013) the studies investigating problem-solving skills (Li & Tsai, 2013) and affective and motivational outcomes were examined more frequently in entertainment game studies (Connolly et al., 2012). Although educational game studies reveal varying degrees of success dependent upon academic topic, learner preferences and participant age (Hays, 2005; Young et al., 2012) impact the development of 21st century skills. Skills relevant to the 21st century are dramatically different from skills the educational system currently values (Squire, 2005). The 21st century learning and innovative skill set is defined as critical thinking, creativity, collaboration and communication (Binkley et al., 2012). Critical thinking skills include scientific reasoning, systems thinking, computational thinking,

decision making and problem-solving. Creativity includes divergent thinking, innovative thinking, originality, inventiveness and the ability to view failure as an opportunity to improve. Collaboration pertains to the ability to work effectively and respectfully with diverse teams, exercise flexibility and willingness to make compromises to accomplish goals and assume shared responsibility. Communication refers to the ability to articulate thoughts and ideas in a variety of forms, communicate for a range of purposes and in diverse environments and use multiple media and technologies. Traditional educational practices often hinder creativity by emphasizing only one correct answer, imposing high-stakes failure and favoring conformity and standardization (Plucker & Makel, 2010). In Thailand, the concept of game-based learning has begun to be used in teaching and learning. Prior studies have shown that the game-based learning group had a knowledge score higher than non-game-based learning (Chaeye & Thammajinda, 2020; Pholtana, 2020; Sornarkas, 2021; Maneejak et al., 2022, Thongpradab et al., 2022). In addition, Sirisawat et al. (2019) studied the effect of 2(CPA) model with a game on critical thinking in nursing student. The result of the study revealed that the student had a higher critical thinking score and was found to be highly satisfactory in all aspects. Lecturers' application of game-based learning to teaching can better integrate the contents of the course for the learners. This technique could contribute more effectively to the learners' improvement of their knowledge of the course contents. Therefore, we can summarize outcomes of game-based learning as shown in the following pictures and details (Fig. 2).

Fig. 2 Summarize outcomes of game-based learning

1. Problem-solving

Yang (2012) stated that a digital game where students designed their own cities using simulation games showed that student's had stronger problem-solving skills. Prior studies revealed that playing games helps improve student attentiveness. As games can move quickly, a student needs to be alert and attentive for extended periods (Yang, 2012). According to the study of Prajankett et al. (2022) about the effect of organizing learning activities based on gamification concept toward problem solving skills of nursing students. After at the end of the determining the outbreak learning activity, the results showed that the nursing students had higher average problem-solving skills than before participating in the activity with statistically significant at the .05 level.

2. Engagement in education

Traditional teaching has provided classroom settings where the engagement level of the students is minimal, often relying on a lecture tactic. GBL can be prepared with a platform that allows students to become engaged and studies show that playing games in the classroom can increase overall motivation. Students become more motivated to learn, pay attention and participate in in-class activities. GBL engages students and enhances studying (Gallegos et al., 2017). They can also be a great classroom management tool, helping to motivate a class. Grech & Grech (2021) indicated that levels of engagement and interaction were higher in the game-based webinar.

3. Immediate feedback

When student's complete tests, assignments, projects, etc., the feedback turn-around time may be too long for them to benefit from the learning. For example, when a mid-unit test is completed, if the feedback is not received until three-quarters of the way through the unit, those students who did not fare well may receive the comment too late to understand the rest of the content (Safeena Beevi & Veragi, 2023). One of the primary benefits of using games is that the feedback can often be instantaneous. This feedback immediately assists the students in their journey to mastering the content and can help them correct their mistakes to further their understanding. Many of these digital games even have a feedback system that can go directly to the teacher, allowing educators to monitor their student's progress and make necessary adjustments.

4. Safe for mistakes

Learning from your mistakes is, at times, one of the best methods of learning how to do something new or

improve your skill set in a particular area (Byrd, 2023). In video games, the ability to make a mistake and be able to try again is a beneficial component to help complete the mastering process. That can help solidify the idea that failure is not only okay but should be embraced. Providing a safe space in the classroom, where students may be afraid to make mistakes, will allow them to be creative and explore this classroom culture, which permits them to make mistakes. Therefore, they can learn without worrying about the opinions of their peers. In addition, students improve their learning and gain confidence in their decision-making as they master the content, which may ultimately progress their classroom skills outside the games. As much as matches in the classroom are about learning a concept, they are also about learning from what goes wrong, which gives students both the problem-solving development we already mentioned and the social-emotional benefits (Castro et al., 2019).

5. Utilize multiplayer based games

Utilization of multiplayer-based games over single-player-based games adds a factor for the students playing: collaboration, whether it's working as a team or against each other, while also adding a higher-level order of thinking to solve the problems that are changing depending on who else is taking part in the games (Tavares, 2022). Depending on the students, some may find motivation to try harder to show their peers they are capable. Perhaps these students will even try to beat their peers at the task or work hard to benefit the team if they are working in a team. For example, you can have the total time of the group add up to see which team performed the fastest by total time or by averages. The effects of game-based learning are principal elements of effective learning environments, which influence students and academics through reciprocity and cooperation communication of expectations and respect for the various learning approaches of students (Urh et al., 2015).

6. Incentives

While providing reinforcement is suggested, frequently, it does not work as well as people may think. Providing incentives sometimes during games may result in students putting in their best effort to complete a match to the best of their ability. Regarding the previous suggestion of using single-player games with speed-based requirements, using these results where the teams can have the option to do something dependent on where they finish can have the students try harder during the game (Anguas-Gracia et al., 2021). For example, educators may provide an incentive such as not needing to help clean the room at the end of the day if they finish with the fastest group time. Utilizing the proper times and incentives leads to students continually putting their best effort into learning and mastering the game themself. All papers reported high levels of students' experience, engagement and motivation in teaching sessions that used game elements. Despite this, levels of engagement and interaction were higher in the game-based webinar (Grech & Grech, 2021). Students perceived game-based learning as more successful at facilitating group and teamwork, improving student relationships and more enjoyable than non-game-based learning (Anguas-Gracia et al., 2021).

7. Focus on deep learning

The use of games promotes deep learning, which involves more critical thinking to solve a task, as opposed to being given a straightforward set of instructions to complete the same assignment; highly engaging video games can often induce a flow state within adolescents which can lead to higher levels of motivation and focus (Castro et al., 2019). Besides providing students with an educational game, this flow state can make them more intelligent in learning and understanding what is happening in the game, promoting more learning. The ability to use serious games in a classroom setting can help students who are actively engaged in the game to achieve that higher level of thinking with problem-solving and become more focused on understanding the content. The GBL teaching improves quality outcomes for students' education. Game-based quizzes and escape rooms were commonly employed when learning clinical skills, developing critical thinking and consolidating learning content (Tavares, 2022). Game-based learning was well accepted by students and seen as more successful at facilitating group work, improving student relationships and more enjoyable than non-game-based learning. GBL was a feasible option for getting the attention of students while achieving deep studying of information.

The use of games in the classroom and assessment

GBL will develop to a new stage, which requires researchers to carry out research to keep pace with the times. For teachers, it provides a guidance on implementing GBL in smart classrooms, because the model proposed in this paper is mainly designed according to the teaching process, teachers can refer to it in different teaching links (Pan et al., 2021).

(1) Before the class, teachers can choose the appropriate games and design teaching activities. Teachers can also design realistic and interactive game-based learning situations.

(2) In the class, teachers can create immersive GBL experience that can evoke thinking, promote learning by exploring through different game activities, as well as developing collaborative capability and to improve interpersonal communication skills. By encouraging presentation and sharing, learners will share their learning results with others and display their works through various content presentation methods in the smart classroom, such as multi-screen display and file transfer between terminals.

(3) After the class, teachers can monitor the online learning process to better enhance learning and improve the quality of teaching. Enhancing personalized guidance, where teachers can find students who have difficulty in learning by viewing and analyzing the student data collected in the learning process. Teachers provide targeted guidance to learners to solve students' learning difficulties. Boosting reflection and improvement, where teachers reflect on the effects of teaching, redesign and improve the deficiencies. Teachers can get enlightenment from the reflection, which can become the experience and basis for teachers to improve their teaching ability.

Game-based learning trends

Game-based learning is a system that is increasing and being implemented in the educational process and reveals clearly in several research papers published over the last decade. In this context, game-based learning is approached via the cognitive and affective dimensions of learning. The users can adopt such games for their intelligible needs and interests and ensure motivation for learning. Game-based learning allows the use of methods: that are consistent with the modern theories on productive learning and encourage active problem-oriented, experimentally interactive and socially mediated access to educational processes according to the current thinking (Vusic' et al., 2018). Researchers suggest that game-based learning in higher education can view advantage tools for developing, implementing, improving and facilitating the student's learning experience. Furthermore, the development a hybrid board game that combines non-digital and digital elements to introduce new game experiences.

Conclusion

This article argues the definition of game-based learning viewpoints of knowledge, motivation and creativity. Outcome of GBL came from various games such as problem-solving and engagement in education. The evidence from advantages of GBL includes helping in retaining learning insights, stimulating imagination, helping in simplifying complex concepts and higher receptivity to learning. We also discussed the type of game that will lead to the design of learning games with the ADDIE model and the game instructional process was designed for evaluation. At present, game-based learning is used in teaching and learning in nursing. Although educational game studies reveal varying degrees of success dependent upon academic topic, learner preferences and participant age which all impact the development of 21st century skills. Lecturers' application of game-based learning to teaching can better integrate the contents of the course for the learners. This technique could contribute more effectively to the learners' improvement of their knowledge of the course content. Further research should investigate the effects of games on affective and behavioral learning outcomes (Ozdemir & Dinc, 2022). The potential of digital games to promote learning is a growing field of education. The increasing use of mobile phones makes it possible to explore digital educational games in outside environments. Because of this, educators can develop a hybrid board game that combines non-digital and digital elements to introduce new game experiences to increase students' motivation and engagement in the learning processes.

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Journal of Food Health and Bioenvironmental Science Publication Ethics

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- It is the duty of the Editors to consider the submitted manuscripts related to field of food, health, biological and environmental disciplines and other related fields. The consideration will be based solely on the content. The ethnicity, country of origin, gender, sexual orientation, political affiliation, or religious belief of authors does not influence the editor's decision.
- Throughout the submission, the editors must not share the information about the submissions to anyone except the authors, reviewers and JFHB staffs.
- Editors must make sure the manuscript has no substantial vested interests authors or affiliated organizations.
- The editorial staff have to assure that the manuscript has been peer-reviewed by at least two reviewers in the field of Food, Health, biological and environmental disciplines or other related field appropriate for each manuscript. The editorial staffs also have to be careful about the copyright Infringement, falsification of data, and plagiarisms. If there is an offense according to the said regulations, the editor must investigate and seek for evidence before consider reject the manuscript.
- In case that the Editors suspect that the manuscript might have been published elsewhere, the editor should investigate and consider reject.
- In case of unethical publishing practices that are later uncovered, the action will still be taken seriously.

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- Reviewers are expect to give constructive and professional comments. Improper criticism must be avoided.
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- If there are conflicts of interests, reviewers should inform the editorial staff. Editors will decide whether the reviewer is appropriate for the manuscript or not.

Author Regulations

- The authors should write the manuscript related to the theme of Food, Health, biological and environmental disciplines. The research manuscript should contained relevant background information, efficient methodology, APA style citation, accurate results, and reasonable discussion.
- The authors should follow the journal guidelines strictly.
- Any opinion or perspective made in the manuscript must be explicitly highlighted as "opinion" or "perspective"
- The authors must be careful and aware that fraudulent information and omission of important information are unethical author behaviors.
- The authors must be able to provide research data if the Editor see needed.
- Authors must reference other works properly. Any work involved in the manuscript also must be well credited.
- The authors must make sure that the manuscript has not been published elsewhere before and is not currently in the publication process in other journals.

- The person must have made significant contributions to the manuscript, participate and give important efficient content during revisions and provide approval for publication in order to be listed as an author. Researchers who do not meet the above criteria should be listed in the Acknowledgements section.

- Author should identify any conflicts of interest that might have influenced the data and/or interpretations of data.

- To make the efficient revision, the authors should respond to all the given critiques and suggestions during the revision.

- If the authors find errors in their works that need to be correct, the author should inform the editors immediately.

Journal of Food Health and Bioenvironmental Science

Vol. 16 No. 1 January – April 2023

Original Articles

Review Article

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