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ANTIFUNGAL ACTIVITY OF *Citrus hystrix* EXTRACT AND ITS APPLICATION AS NATURAL FOOD PRESERVATIVE

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Running title: Antifungal activity of *Citrus hystrix* DC as food preservatives

ABSTRACT

Traditional ready to eat foods, such as sticky rice cake, are easily contaminated by spoilage pathogens during their storage. Hence, this study aims to evaluate the effect of *Citrus hystrix* extract for reducing spoilage pathogens in sticky rice cake during storage. The tested sticky rice cake was formulated with *Citrus histrix* extract at varied level of concentrations of 0.65%, 1.26%, and 1.82% (w/w). Treated samples were stored at room temperature for 28 days and evaluated periodically for their microbial activity (total plate count), thiobarbituric acid reactive substances (TBARS), and sensory analysis. The *Citrus histrix* extract was also examined for its antifungal activity against *Penicillium sp.* and *Aspergillus nidulans* prior to formulation. Results exhibited a significant advantage of the addition of extracts to sticky rice cake. All extract levels effectively eliminated the spoilage microorganism until 28 days storage and significantly lowered the TBARS values. Physico-chemical properties of sticky rice cake including pH, water activity, and moisture content were equal among all formulated samples and slightly different at 1.82% (w/w) extract level. Moreover, the addition of *Citrus hystrix* extract up to 1.82% did not affect the acceptability of sticky rice cake on the sensory attributes as compared to the control (P>0.05).

Keywords: Antifungal, *Citrus hystrix*, sticky rice cake, natural preservative

INTRODUCTION

In the recent years, food safety issues have become one of the main public health concerns. The spoiled food is unacceptable for human consumption due to alteration in sensory attributes such as taste, colour, appearance, odor, and texture (Holley & Patel 2005). Some methods widely used to protect microbial spoilage of food commodities is the addition of synthetic or natural preservatives, which directly supplemented to the foods or incorporated in the food packages (Brul & Coote 1999). Recently, there has been a worldwide effort to minimize the use of chemical preservatives since consumer preferences are inclined towards more natural and healthier products. Consequently, the consumer's inclination for foods deprived of chemical preservatives has led to the discovery of new natural antimicrobial and antioxidant preventing agents (Serra *et al*. 2008).

Natural preservatives such as essential oils, flavonoids, phenolic compounds, and microbial metabolites are the chemical agents derived from plants, animals, and microbes that could preserve food by fighting against fungi and food borne bacteria (Prakash *et al*. 2014). They prevent the decomposition of products by inhibit microbial growth, oxidation and certain enzymatic reactions occurring in the foodstuffs (Singh *et al*. 2010). *Citrus hystrix* (family Rutaceae), commonly known as kaffir lime, is a tropical herb distributed in Southeast Asia. The useful parts of *C. hystrix* are the fruit, leaves, and peel. Its leaves are aromatic and used as a spice and for various flavoring purposes. The essential oil of *C. hystrix* was characterized by high contents of terpinen-4-ol (13.0%), a-terpineol (7.6%), 1,8-cineole (6.4%), and citronellol (6.0%). Previous studies reported that *C. hystrix* oil was effective for antioxidant, repellent, and antiviral (Waikedre *et al*. 2010). Its oil has antibacterial activity against 20 serotypes of Salmonella and five species of other enterobacteria (Nanasombat & Lohasupthawee 2005).

Sticky rice cakes are popular dessert in Asian countries, particularly in Indonesia. Sticky rice cakes can be made in different base matters including sticky or glutinous rice, sugar (palm, brown, and cane), and some other ingredients such as variety of beans, coconut, and sesame seeds. The most popular methods to prepare these foods are by way of steaming, frying, and boiling (Lee *et al*. 2009). Sticky rice cake is one of ready to eat foods which is commonly stored in room temperature. In the state of California, USA, Korean rice cakes are allowed to be sold at room temperature for up to 24 hours after production by listing the date and time of manufacturing (California State Legislature 2016). Most literatures reported that sticky rice cakes can be kept for up to a few days of storage at room temperature. In addition, there have been many investigations on how to maintain the microbiological quality of these products. On this basis, this present study aims to evaluate the potential of *C. hystrix* extract for reducing spoilage pathogens in ready to eat food, sticky rice cake, during storage.

MATERIALS AND METHODS

Preparation of leaves extracts

C. hystrix leaves were purchase from Beringharjo market, Yogyakarta, Indonesia. The leaves were washed, dried, and grinded into powder. Then, it was turned into aqueous extract by homogenizing of 250 g of the material with 2500 mL of distilled sterile water, followed by sonication (for 60 min three times). The resulted liquid was filtered and evaporated to get concentrated extract. The extract was further used in formulation for food preservative.

Evaluation of antifungal activity

Three food spoilage microorganisms were isolated from sticky rice cake and identified as *Penicillium sp, Aspergillus nidulans,* and *Rhizopus stolonifer*. Those fungi were then isolated and further used as the test organisms. The plates containing three extract concentrations, including control sample were inoculated in the core of the plate by spotting the 8 mm in diameter of fungal species until round inoculums were formed. Inoculation was performed in four replications with two inoculums per plate. After inoculation, Petri plates were closed properly and incubated at 27 °C. The evaluation of antifungal activity was carried out by measuring of the radial growth of the mycelium (in diameter) in each plate during 3 days for *Aspergillus nidulans* and *Rhizopus stolonifer* and 7 days for *Penicillium sp* in the presence of extracts. The antifungal activity (AFA) was calculated by the following equation:

$$AFA\ (\%) = (GC - GT) / GC \cdot 100$$

It is noteworthy that AFA is antifungal activity (%), GC is colony diameter on the control plate (mm) and GT is colony diameter on the test plate (mm) (Mori *et al*. 1997).

Preparation of extract granule and sticky rice cake

The concentrated extract of *C. hystrix* leaves was formulated with lactose through a granulation process with a ratio of 1: 4 (w/w). The extracts were homogeneously mixed with lactose fillers to produce a convenient wet mass. The wet mass was passed through a sieve with 30 mesh size. The obtained granules were dried at 60 °C for 36 hours to reach less than 2% of moisture content. Dried granule was then passed back through a sieve with a 30 mesh size and stored until it is used as food preservative.

The resulted granule was then applied on the processing of sticky rice cake with varied concentration (0.65%, 1.26%, and 1.82%). Granule was mixed with the sticky rice cake dough in a few minutes before product was completely cooked, as the temperature was getting lower. The product was packed in pieces of small plastic.

Physico-chemical properties of sticky rice cake

Moisture content was calculated using moisture balance (AND MX50, Tokyo, Japan). Water activity was determined by Kjeldahl steam distillation (JP Selecta Pro-Nitro S 4002851, Barcelona, ES). The pH value was measured in pH meter (Eutech PC700, NY, US).

Microbial analysis of product

Four samples of the tested food product including control were analyzed after 0, 7, 14, 21, and 28 days of storage. Total plate count (TPC) was determined following the incubation at 37 °C for up to 28 days, on Plate Count Agar (PCA, Merck). Twenty five grams of sample were dissolved in 225 ml of 0.85% NaCl solution, and then mashed using a stomacher. The prepared samples were serially diluted tenfold addressed to minimize the number of microbes in the sample solutions. The bacteria were isolated by pour plate methods using10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions. The colonies were counted using colony counter after 24 hrs and 48 hrs incubation and the results were expressed as cfu/ml.

Aerobic plate count was determined by the spread plate technique using standard methods with some modification. All yeast and mould counts were done in triplicate, using Potato Dextrose agar (PDA) medium (Merck, Germany), supplemented with 0.01 % chloramphenicol (Merck, Germany). PDA plates were incubated for up to 5 days at 30 °C. The number of visible colonies on Yeast and Mould Count Plate was read after 3 days and 5 days.

TBARS determination

The tested food products were analyzed after 0, 14 and 28 days of storage. Lipid stability was evaluated using the thiobarbituric acid reactive substances (TBARS) index according to a method reported by Targladis *et al*. (1960) with slight modification. Ten grams of sticky rice cake sample in the distilled water (50 mL) were minced using a blender (Philips, England). The minced product was filtered and transferred to a distillation system and steam distillation carried out with 4N HCl (2.5 mL). The distillate (2.5 mL) was reacted with a 0.02 M thiobarbituric acid solution (2.5 mL), incubated in a boiled water bath for 35 min, and then cooled until it reachs room temperature. Absorbance at 528 nm was measured using a UV Vis Spectrophotometer (Hitachi HALO RB-10). Thiobarbituric acid reactive substances values were calculated from a standard curve and expressed as mg MDA/kg sample.

Sensory analysis

The sticky rice cake characteristics was frequently evaluated in terms of odor, tastes, colour, texture, and overall acceptance (Meilgaard *et al*. 1991). The 20 untrained taste panellists assess the sensorial properties every 7 days during products storage. Sensory evaluation scores were determined using a 7-point Hedonic scale ranging from 1 to 7. The same superscript symbols in the sticky rice cake score indicate that sample are not significantly different at a significance level of 95%.

Statistical analysis

Mean values and standard deviations were calculated from the data obtained from triplicate experiments. One-way Analysis of Variance (ANOVA) with a significant level of 95% test was used to determine the significant differences between variables.

RESULTS AND DISCUSSION

Antifungal activity of *C. hystrix*

The antifungal activity (AFA) of *C. hystrix* extract has been evaluated towards the isolated spoilage fungi growth in tested sticky rice cake that was identified as *Penicillium sp* and *Aspergillus sp*. The *in vitro* results showed that the selective antifungal activity of *C. hystrix* extract against *Penicillium sp.* and *Aspergillus nidulans* were 33.53 ± 31.67% and 40.22 ± 11.08%, respectively. Meanwhile, there was no antifungal activity observed against *Rhizopus stolonifer*. Since *C. hystrix* extract exhibited remarkable antifungal activity, thus the extract was further tested in a traditional sticky rice based dessert, sticky rice cake. Warm climate and avoiding refrigeration are the main causes of high level contamination in sticky rice due to spoilage of microorganism. To the best of our knowledge, the effect of *C. hystrix* leaf extracts on the microbiological stability of food products containing sticky rice had not been studied previously.

Physico-chemical properties of sticky rice cake

Abundant nutrients, high water activity, and nearly neutral pH make sticky rice cake a good medium for microbial growth and categorized as products that require temperature control. Therefore, the preservative additive might be necessary because this product is normally stored through un-refrigeration method. Water activity is one of the variables that contribute to the reduced shelf life of a food product. Water can be retained and trapped inside the pores of food products under the activity of water which should be removed. The water activity of all samples was ranged 0.68 to 0.78 (see Table 1). This range is relatively lower than the minimum growth requirement for several pathogen bacteria, such as *S. aureus* (0.86), *B. subtilis* (0.95), *E. coli* (0.96), *Salmonella spp*. (0.96), and *Psedomonas spp*. (0.97) (Molan 1992). According to Filtenborg *et al*. (1996), the spoilage is due to species enduring in a lower water activity (0.95). High lactose concentration (1.82% extract level) led to the reducing of water activity as reported by Mundo *et al*. (2004).

Table 1 Physico-chemical properties of sticky rice cake formulated with *C.hystrix* extract

Physico-chemical properties	**Level of extracts (%)**				
	Day	Control[a]	0.65	1.26	1.82
Water activity (a_w)	0	0.78	0.78	0.78	0.76
	14	0.76	0.70	0.70	0.68
	28	0.77	0.77	0.76	0.75
pH	0	6.21	6.07	5.56	6.01
	14	6.18	6.22	6.05	5.88
	28	6.27	6.26	5.93	5.76
Moisture content (%)	0	27.83	25.24	24.86	27.28
	14	20.15	19.14	21.05	20.77
	28	18.27	18.07	18.30	19.14

Control : product in the absence of extract.

Total Plate Count (TPC)

Most (sticky) rice-based desserts and snacks are soft and elastic, but during the storage they become hard due to retrogradation in room temperature. Hence, it is not suggested to store them using refrigeration (Morris 1990). They are ready to eat food as they contain some components that are already cooked and do not need further heating prior to consumption, so the microbiological quality of sticky rice cake is very important. Microbiological purity is an important quality criterion in the food products, with a limit for the number of microbes as stipulated in applicable regional law. The average number of microbial colonies in sticky rice cake after 28 days storage is shown in table 2.

Table 2 Total bacteria of sticky rice cake formulated with *C.hystrix* extract

Storage duration (days)		**Bacterial colonies (cfu/ml)**			
		Control	A	B	C
0	24 h	0.51×10^4	0.93×10^4	9.75×10^4	1.10×10^4
	48 h	0.36×10^4	0.66×10^4	10.45×10^4	0.71×10^4
7	24 h	7.90×10^4	0.64×10^4	1.02×10^4	1.29×10^4
	48 h	6.45×10^4	0.97×10^4	0.99×10^4	1.02×10^4
14	24 h	1.09×10^4	0.31×10^4	0.19×10^4	1.83×10^4
	48 h	1.04×10^4	0.46×10^4	0.22×10^4	2.89×10^4
21	24 h	0.34×10^4	0.20×10^4	0.10×10^4	0.20×10^4
	48 h	0.43×10^4	0.31×10^4	0.15×10^4	0.30×10^4
28	24 h	0.20×10^4	0.20×10^4	0.15×10^4	0.20×10^4
	48 h	0.41×10^4	0.25×10^4	0.20×10^4	0.60×10^4

Control: product in the absence of extract.; A: product formulated with 0.6% extract; B: product formulated with 1.26% extract; C: product formulated with 1.82%.

Total plate count (TPC) value of sticky rice cakes formulated with *C. hystrix* extract were ranged from 0.1×10^4 to 10.5×10^4 cfu/ml. The addition of uneven extracts during mixing process resulted in a significant increase in the TPC value during storage time. All samples were classified as good or acceptable level of microbiological quality, which are $<10^5$ cfu/g as the guideline for ready to eat foods (NSW Food Authority 2009). Moreover, testing results for products showed that the addition of *C. hystrix* extract as antibacterial agent was effective to reduce the microbial growth on weeks observation compared to the control. However, the better capability was exhibited by the 1.26% extract than 1.82% extract. Lee *et al*. (2009) reported that the addition of 1 or 3% green tea or rosemary to rice cakes gave no significantly affect to the number of microbial properties.

Conversely, on three days storage, the growth of *B. cereus* and *S. aureus* could be inhibited at room temperature. The main causes of ready to eat food poisoning are microbial pathogens such as *S. aureus* (Huong *et al.* 2010).

Yeast and mould counts

C. hystrix extract improved the microbial quality of sticky rice cake particularly on the level of 1.82 % extract over 14 days storage compared to the control (see Table 3). However, after 28 days of storage, the sticky rice cake formulated with 1.26% extract gave a better shelf life toward yeast and mould growth with the number of 26×10^2 cfu/ml compared by control. Mould growth in this formula is an indication of high moisture content in the product. The higher extract was formulated in the sticky rice cake, the higher amount of lactose was added. Consequently, it will easily absorb water, thus allowing the mould to grow. It was shown by the moisture content of product formulated with 1.82% which is higher than those of other formulas (19.14%).

Table 3 Total yeast and mould of sticky rice cake formulated with *C. hystrix* extract

Storage duration (days)		**Yeast and mould (cfu/ml)**			
		Control	A	B	C
0	3 days	2.0×10^2	2.0×10^2	0.4×10^2	0.3×10^2
	5 days	3.0×10^2	4.0×10^2	3.0×10^2	2.0×10^2
7	3 days	3.8×10^2	3.2×10^2	1.4×10^2	1.4×10^2
	5 days	4.1×10^2	4.1×10^2	4.0×10^2	3.0×10^2
14	3 days	10×10^2	1.0×10^2	1.0×10^2	0.9×10^2
	5 days	4.0×10^2	5.0×10^2	2.0×10^2	1.8×10^2
21	3 days	9.0×10^2	8.0×10^2	1.0×10^2	4.0×10^2
	5 days	12×10^2	11×10^2	3.0×10^2	11×10^2
28	3 days	34×10^2	25×10^2	21×10^2	27×10^2
	5 days	39×10^2	28×10^2	26×10^2	32×10^2

Control : product in the absence of extract.; A : product formulated with 0.6% extract; B : product formulated with 1.26% extract; C : product formulated with 1.82%.

Moulds are filamentous fungi with branching hyphae, multi-cellular, generally aerobic and grow at a pH range of 3 to 8. The spores can tolerate harsh environmental conditions but sensitive to heat treatment (Pal 2007). The formulation of sticky rice cake with *C. hystrix* extract slightly decreased the pH value of product. Moreover, the highest acidity of sticky rice cake was reached during the 28 days storage on the sticky rice cake formulated with 1.82% extract. The high acidity favours the growth of moulds (Loureiro & Querol 1999). The addition of extract effectively eliminated the spoilage microorganism until 28 days storage. After 28 days, the product was fully contaminated by moulds. As reported by Ji *et al.* (2007), microorganism grows significantly over time in rice cakes and the product would completely spoiled shown by altering the colour, texture, and flavour after three days of storage at room temperature. Moulds commonly grow on all kinds of food such as cereals, meat, milk, fruit, vegetables, nuts, and fats. Mycotoxins, the toxic secondary metabolites toward vertebrate animals in small amounts through natural route, are produced during growth of moulds on food products. Mycotoxins from Penicillia normally grow in cereals kept in tropical countries with warm climate and high moisture including viridicatumtoxin (*P. aethiopicum*), citrinin (*P.citrinum*), cyclopiazonic acid, patulin and roquefortine C (*P. griseofulcum*) and secalonic acid D (*P. oxalicum*) (Frisvad & Filtenborg 1989).

TBARS analysis

Sticky rice cake, containing coconut milk, renders a major problem associated with the producing of off-odors due to free radical induced lipid oxidation. The *C. hystrix* extract was added to a processed sticky rice cake to improve sensory attributes and control oxidation reactions. The effect of *C. hystrix* extracts after processing and during room temperature storage is shown in Table 4.

Table 4 Effects of *C. hystrix* extract on oxidative stability (TBARS values) of sticky rice cake

Storage duration (days)	**TBARS index (mg MDA/kg)**			
	Control	A	B	C
0	0.45	0.22	0.39	0.24
7	0.33	0.39	0.39	0.49
14	0.22	0.16	0.41	0.17
21	0.67	0.47	0.33	0.36
28	0.18	0.19	0.15	0.12

Control : product in the absence of extract.; A : product formulated with 0.6% extract; B : product formulated with 1.26% extract; C : product formulated with 1.82%.

Adding *C. hystrix* extract to sticky rice cake rather reduced the TBARS values during 28 days storage with no significant difference between the three levels extract formula. Addition of herbal extracts as antioxidant supplementation in some foods was effective in controlling lipid oxidation during storage (Formanek *et al*. 2001; Park *et al*. 2016). Moreover, the pH of food condition gives influence in the activity of oils. At low pH, the hydrophobicity of some essential oils contained in *C. hystrix* increase and it is subjected to partition in the lipid phase of the food, thus affecting in reducing oxidative stability.

Sensory analysis

The organoleptic analysis was carried out after a week in storage to evaluate the influence of *C. hystrix* extract on the acceptability of product by customer (see Table 5).

Table 5 Sensory evaluation of sticky rice cake formulated with different levels of *C. hystrix* extract

Level of extracts (%)	**Attributes**				
	Colour	Odor	Taste	Texture	Overall acceptability
Control (0)	5.89[ab]	6.33[b]	6.67[b]	5.94[b]	6.56[b]
0.65	5.50[a]	5.50[ab]	5.61[a]	4.89[a]	5.78[a]
1.26	6.06[ab]	5.39[a]	5.67[ab]	5.78[ab]	5.94[ab]
1.82	6.44[b]	5.89[ab]	5.83[ab]	6.33[b]	6.67[b]

[a,b,ab] Mean values in the same column with different letters are significantly different at $p < 0.05$

The addition of *C. hystrix* extract did not reduce the acceptability of sticky rice cake on the sensory attributes as compared to the control sample, sticky rice cake with no addition of extract. Statistically, there is no significant difference of acceptance among products formulated with 0.65%, 1.26%, and 1.82% extracts. However, the sensory test should be performed every week until 28 days storage in order to observe the alteration of acceptance level. Most plant extracts do not give the effect on the acceptability of sticky rice cake as long as the appropriate concentration is used. Irradiated and freeze-dried green tea leaf extracts formulated in raw and cooked pork patties were reported giving no influence on the physical and sensory properties (Jo *et al.* 2003). The results of our study indicated that sticky rice cake formulated with *C. hystrix* extracts exhibited sensory stability similar to the control on one-week storage.

CONCLUSION

Addition of *C. hystrix* extract in food formulation could reduce the microbial growth as well as extending the shelf life of sticky rice cake. This suggests that *C. hystrix* enhances the antifungal activity over time. The increasing level of *C. hystrix* extract up to 1.82% of sample (w/w) is still acceptable in overall sensory attributes. Thus, sticky rice cake with *C. histrix* extract adds potential value to efforts to provide the quality and healthy food options in Indonesia.

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