In Vitro Antifungal Efficacy of White Radish (Raphanus sativus L.) Root Extract and Application as a Natural Preservative in Sponge Cake

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Date Submitted: 2019-11-24

Keywords: sponge cake, shelf-life study, antifungal activity, Raphanus sativus L.

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Record Type: Published Article

Submitted To: LAPSE (Living Archive for Process Systems Engineering)

Citation (overall record, always the latest version):	LAPSE:2019.1138
Citation (this specific file, latest version):	LAPSE:2019.1138-1
Citation (this specific file, this version):	LAPSE:2019.1138-1v1

DOI of Published Version: https://doi.org/10.3390/pr7090549

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Received: 10 July 2019; Accepted: 6 August 2019; Published: 21 August 2019

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Keywords: *Raphanus sativus* L.; antifungal activity; shelf-life study; sponge cake

1. Introduction

Raphanus sativus L., commonly referred to as radish or daikon, is a cruciferous vegetable that belongs to the Brassicaceae family and finds a myriad of applications in the daily diet and traditional medicine of Eastern societies. The radish is one of the most consumed vegetables in Asian countries, including Vietnam, China and Japan, and is regarded as an important ingredient in the treatment of liver and respiratory diseases [1,2]. Biological activities of radish are diverse and have been ascertained in many investigations, which were well summarized in a past review [3]. In brief, the radish extract was shown to exhibit inhibitory effect on growth of G+, G" bacteria, antifungal, immunological, antioxidant, anticancer and antiviral properties. The antibacterial activity is mostly due to the presence of cysteine-rich peptides (Rs-AFP1 and Rs-AFP2), RAP-1 and RAP-2 protein, cafeic, ferulic and phydroxybenzoic acid. In addition, it was found that four types of the glucosinolate compound, including glucoraphenin, dehydroerucin, glucobrassicin, and glucoerucin are responsible for the pungency of *Raphanus sativus*, predominate in roots of radish [4]. Glucosinolate has been shown to take part in promoting enzymatic activities involving removal of carcinogens and preventing aging in the human body.

Different parts of the radish plant show varying degrees of biological activities [5,6]. While seeds and aerial parts of *Raphanus sativus* have been long known for strong antifungal and antibacterial activity due to the presence of the raphanin compound, to date, current evidence has only documented moderate inhibitory effects of the *Raphanus sativus* root against various bacteria species only and thus, making data with regard to antifungal activities of *Raphanus sativus* uncomprehensive [7–10]. For example, in a previous in vitro study involving 52 selected food-borne bacteria species, methanol extracts from white skin *Raphanus sativus* roots were shown to inhibit the growth of 11 species (*Arthrobacter atrocyaneus*, *Corynebacterium ammoniagenes*, *Enterobacter hormaechei*, *Kocuria rosea*, *Neisseria subflava*, *Pantoea agglomerans*, *Proteus vulgaris*, *Psychrobacter immobilis*, *Shigella dysenteriae*, *Bacillus sphaericus* and *Corynebacterium flavescens*) [11]. This result was further extended in another similar study where growth-inhibitory effect of radish root juice was determined against five other microbial species including *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli* with minimum inhibitory concentrations (MIC) ranging from 0.078 to 0.625 mg/mL [12]. However, those reported antibacterial effects were not as high as those exhibited by standard drugs, which are respectively maxipime and ampicillin in the two studies.

Apart from the cysteine-rich peptides, the antibacterial activity of the taproot of *Raphanus sativus* might be attributed specifically to its content of flavonoids, which figure for various health-beneficial effects and strong antimicrobial properties [3]. The inhibition mechanism of flavonoids against microbial growth was implied in a review proposing that highly polar phenolic compounds, including flavonoids, tannins, and quinones, could bind to and thus interfering with the conformation of ABC transporter proteins in pathogenic fungi and microbes [13,14]. In Indian *Raphanus sativus* roots, the total flavonoid content reached 43.5 mg CE/100 g fresh mass, which was comparable to those found in Indian apples and ladyfinger fruits, respectively at 48.5 and 48.2 mg CE/100 g fresh mass [15]. This was supported by results of another study where flavonoids and alkaloids were consistently found in *Raphanus sativus* root peel extracts regardless of employed solvent and extraction method [16]. In terms of flavonoid composition in *Raphanus sativus* root, it was revealed that radish root contains four major types of flavonoids including kaempferol, luteolin, quercetin and apigenin respectively at 32.3, 19.5, 5.2 and 2.2 mg/kg fresh weight [17].

It is a common extrapolation in previous antimicrobial and antioxidant assays that *Raphanus sativus* taproots could act as a promising ingredient for manufacture of bio-preservatives used in foods. However, to our knowledge, it is difficult to comprehend this justification in absence of relevant results on antifungal activity. In addition, to permit feasible production of health-safe additives, optimization of flavonoid content accruing from the extraction process of the radish root as well as preservability and palatability evaluation of food supplemented with radish-derived preservatives should be carried out. Therefore, the objective of in this study is three-fold. First, we optimize the total flavonoid content in the extracts of the *Raphanus sativus* roots with respect to different experimental parameters. Second, the antifungal activity against *Aspergillus flavus* NBRC 33021, *Aspergillus niger* NBRC 4066, *Aspergillus clavatus* NBRC 33020, and *Fusarium solani* NBRC 31094 fungi of the obtained *Raphanus sativus* taproot extracts will be determined via well diffusion assays. Third, to assess the suitability of the extracts in food applications, we evaluate preservability, sensory characteristics and several important microbial indicators of a sponge cake formula incorporated with the dried extract of the *Raphanus sativus* root. The results are expected to aid further development of natural preservatives derived from abundant and inexpensive plant materials.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Materials

White radish roots were collected from the farming field of Tran Gia Company, a major vegetable provider situating in Dalat city, Lam Dong province, Vietnam. Obtained roots were of the first

generation (F1) weighing around 200–300 g/root, having the diameter of 3.5 ± 0.5 mm and length of 17.5 ± 2.5 cm. Materials were pretreated by washing under tap water and then sliced and dried at 50 °C to reach the final moisture level of 6–7% (w/w). Subsequently, the dried slices of white radish were ground to a powder and sieved through a sifter (40 mesh). Finally, they were stored in sealed bag and kept in a freezer at −20 °C.

2.1.2. Ingredients for Sponge Cake

Ingredients include fresh whole eggs (Ba Huan Joint-Stock Company, Ho Chi Minh City, Vietnam), medium flour (Interflour Group Pte Ltd., Ho Chi Minh City, Vietnam), powdered refined white sugar fine (Ninh Hoa Sugar Joint Stock Company, Khanh Hoa province, Vietnam), vegetable oil (Cargill Inc., Kuala Lumpur, Malaysia), sorbitol liquid (Roquette, Lestrem, France), cake-gel emulsifier (Rikevita, Johor, Malaysia), whole milk powder (Fonterra, Auckland, New Zealand), salt (Tan Thanh, Ho Chi Minh City, Vietnam), xanthan gum (ADM, Chicago, IL, USA).

2.1.3. Culture Media and Microorganisms

Sabouraud dextrose agar (SDA) was purchased from Himedia Laboratories, Mumbai India. Commercial antifungal agent of Natamycin was purchased from Handary (Uccle, Belgium).

The mold isolates of *Aspergillus flavus* NBRC 33021, *Aspergillus niger* NBRC 4066, *Aspergillus clavatus* NBRC 33020 and *Fusarium solani* NBRC 31094 were obtained from stock cultures of the Institute of Microbiology & Biotechnology, Vietnam National University, Hanoi, Vietnam.

2.1.4. Photocatalytic Degradation of Rhodamine B

All chemicals and solvents were of analytical grade. The reagents used were Trolox (Sigma-Aldrich 23,881-3, St. Louis, MO, USA), (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), was used as anti-oxidant. ABTS (Sigma-Aldrich, A1888, St. Louis, MO, USA)–2,2′-Azino-bis (3-Ethylbenzthiazoline-6-sulfonic acid) and potassium persulfate $K_2S_2O_8$ (Sigma-Aldrich, P5592, St. Louis, MO, USA) were used to prepare a solution containing the radical cation $ABTS^{\bullet+}$. Gallic acid (3, 4, 5, trihydroxybenzoic acid, $C_6H_2(OH)_3$-COOH, MW = 170/12), quercetin ($C_{15}H_{10}O_7$), sodium bisulfite ($NaHSO_3$), and acetate (CH_3COOK) were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Method

2.2.1. Preparation of White Radish Extract (WRE)

Figure 1 illustrated the preparation process of WRE. The root powder was extracted via solvent extraction method with ethanol 70% (*v*/*v*), ratio of substrate and ethanol: 1/20 g/mL for 4 h at 60 °C. After extraction, the mixture was filtered thrice through Whatman No.1 paper. The filtrate was evaporated under vacuum to a constant weight to obtain a crude, flavonoid-rich paste. Prior to spray drying, this crude paste was mixed with maltodextrin 4% (based on the dry matter). To obtain white radish root extract (WRE) with a moisture of lower than 6.0% (w/w), the temperature for spray drying was maintained at 160 °C. Finally, WRE were packed tightly in a thermoplastic low-density polyethylene bag and avoid direct light until further use.

2.2.2. Preparation of Sponge Cake

The sponge cake formula was produced by a standard all-in procedure, as illustrated in Figure 2 [18]. All ingredients were mixed in a mixer (Kenwood KM250, Kenwood Ltd., Woking, UK). For the control sample, 1300 ppm of potassium sorbate was added. For the testing sample, this preservative was replaced by 30 mg/mL of WRE. After mixing, the dough was transferred to aeration machine to create a foam structured dough. The specific gravity of the obtained dough was approximately 0.47–0.49 and the temperature was around 28–30 °C. Prior to baking, the dough was transferred into tanks and was pumped into molds through forming nozzles. An automatic oven (Dong Yang Dynamics) with

baking functionality was employed with setting profiles: 170 °C for 16 min for oven zone 1, 190 °C for 5 min for oven zone 2; 220 °C for 3 min for oven zone 3, 1 min for damper, total baking time of 23 min. The cake after cooling is injected with a custard cream with a weight of 8.0–8.5 g/piece. To preserve the product, each cake was sprayed with 0.3 ± 0.05 mg of alcohol solution 30% (*v*/*v*) onto their surfaces. Finally, the products were packed under tight conditions and prevented from exposure to air.

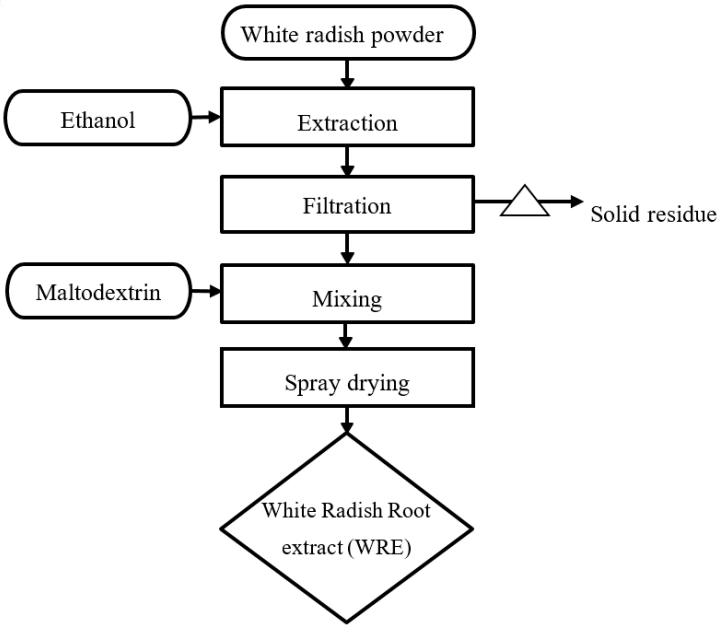

Figure 1. The procedure for the preparation of White Radish Extract (WRE).

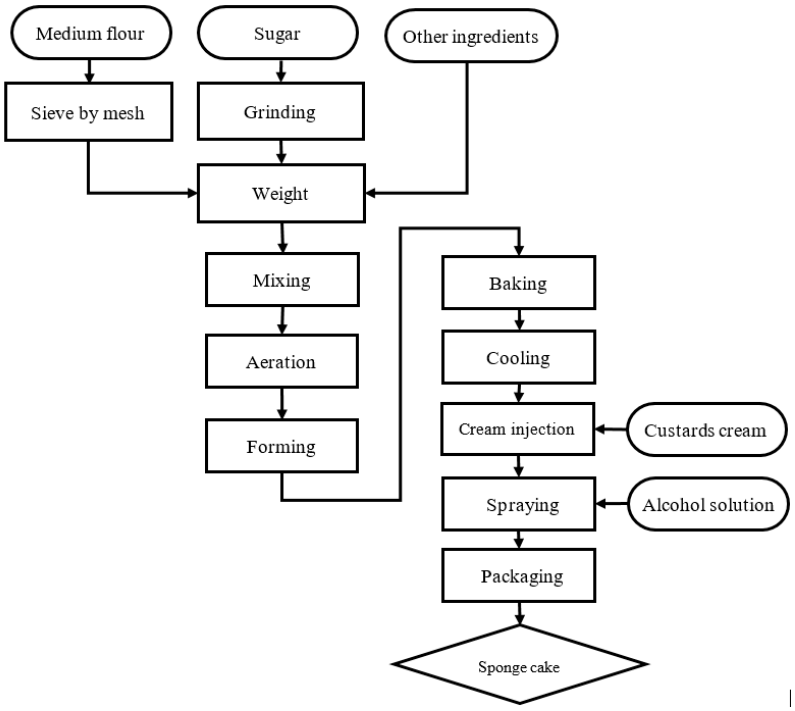

Figure 2. The procedure for preparation of sponge cake.

2.2.3. Determination of the Nutritional Components in White Radish Materials

Moisture content, protein content, lipid total and ash total in the roots were determined respectively in accordance with TCVN 4326:2011, TCVN 4328:2007 (Kjeldahl), TCVN 4331:2001 and TCVN4327:2007.

2.2.4. Determination of the Total Flavonoids

The total flavonoids were determined by the aluminum chloride calorimetric method using quercetin as a standard [19]. The stock quercetin solution was prepared by dissolving 5.0 mg of quercetin in 1.0 mL of methanol. The stock solution was serially diluted produce standard solutions with varying concentrations of quercetin (5–200 µg/mL). An amount of 0.6 mL of diluted standard quercetin solutions or 0.6 mL of WRE was separately mixed with 0.1 mL of 10% aluminum chloride. After mixing, the mixture was incubated for 60 min at room temperature. The contents of flavonoids were calculated from an absorbance of the reaction mixtures at 415 nm with a Varian UV-Vis spectrophotometer as in an equation below:

$$\mathrm{TFC} = \frac{\mathrm{a} \times \mathrm{V} \times \mathrm{n} \times 10^{-3}}{\mathrm{m}} \quad (1)$$

where total flavonoid content (TFC), a, V, m and n are total flavonoid content (mg QE/g), quercetin content derived from the standard curve (ppm), total volume of solution (mL), sample weight (g) and dilution factor, respectively.

2.2.5. Determination of the Antioxidant ABTS

Radical scavenging activity (ABTS) was determined based on a reduction of ABTS radical cations by antioxidants in extracts [20]. The mixture of WRE and distilled water directly reacted with $K_2S_8O_2$ 2.45 Nm (ratio 1:1) in the dark for 12–16 h at ambient temperature. Afterwards, 3 mL of ethanol solution was added and the mixture was allowed to stand for 6 min. The absorbance was measured at a wavelength 734 nm. Changes in optical density (OD) were calculated as follows.

$$\%OD = \frac{A_{sample}}{A_{control}} \times 100 \quad (2)$$

A_{sample}: absorbance of test sample, $A_{Control}$: absorbance of blank.
ABTS radical scavenging activity was calculated as:

$$\mathrm{ABTS(mgTEAC/g)} = \frac{\mathrm{a} \times \mathrm{V} \times \mathrm{n} \times 250.29 \times 10^{-6}}{\mathrm{M}} \quad (3)$$

a: Trolox content (µM) calculated from the calibration curve. V: Total volume from solution extract (mL), M: Dry matter mass (g), n: Dilution factor, 250.29: Trolox molecular weight.

2.2.6. Preparation of Inoculum

Stock inoculum suspensions were prepared as described in the NCCLS M38-P document [21]. Suspensions were collected from 7-day-old cultures grown on potato dextrose agar slants and adjusted spectrophotometrically to optical densities ranging from 0.09 to 0.3 (82 to 60% transmittance). The stock suspensions consisted of mostly conidia and were diluted at the ratio of 1:50 in the mold medium. The final conidium counts in tested stock inoculum suspensions ranged from 0.5×10^6 to 4.5×10^6 CFU/mL (1000 to 9000 CFU per the inoculated well) [22].

2.2.7. Antifungal Susceptibility Testing by Disk Diffusion Method

The employed microdilution methods were adapted from a described method [21]. In brief, the stock suspension of each fungal species was inoculated onto a petri disc with six wells with varying concentrations of fungi. The result was record after 48 h in incubator at 35 °C as diameter

of the inhibition zone corresponding to *Aspergillus flavus*, *Aspergillus clavatus*, *Aspergillus niger*, and *Fusarium solani*.

2.2.8. Determination of Minimal Fungicidal Concentration (MFC)

The in vitro minimum fungicidal concentration (MFC) was defined as the lowest drug concentration at which colonies were completely inhibited (approximately 99 to 99.5% killing activity) [22]. For each fungal species, a 7-well petri disc filled with WRE at varying concentrations from 2.5–50 mg/mL was compared with a similarly inoculated control disc filled with natamycin with concentration ranging from 0.025–0.8 mg/mL. The plates were incubated at 35 °C and the results were recorded after fungal growth was seen in the disc.

2.2.9. Water Activity Method

Absolute humidity is the measured amount of water in grams per cubic meter of air. Relative humidity is the amount of moisture in the air compared to the maximum amount of moisture the air contains at a given temperature. It is measured as a percentage of saturation. Theoretically foods can have a water activity between 0 (bone dry) to 1 (dry water). In this study, using the water activity meter of Aqua Lab Pre at 20 °C. Water activity is defined in the following formula: $a_w = p/p_0$ = ERH/100 where ERH stands for equilibrium relative humidity (%).

2.2.10. Sensory Evaluation Method

The employed sensory evaluation was in accordance with difference from control (DFC) method [23]. To be specific, 18 panelists were recruited and each panelist is presented with the control sponge cake product and several other batches coded with three-digit numbers. The panelist assessed the control product first and was then asked to determine how different each individual sample was when comparing with the control on a numerical scale from 1 to 6. Of which, the scale of 1 indicated no difference and 6 was a marked difference observed between the control cake and treated cake [24].

Accepted:

6–Identical to the reference cake
5–Slight difference was observed
4–Moderately different
3–Distinctly different, but acceptable.

Rejected:

2–Distinctly different (partial or complete loss of aroma of the cake)
1–Extremely different, not consumable and considered spoiled.

2.2.11. Texture Analysis Method

Measurement of the firmness of sponge cake by Stable Micro Systems with texture analysis setting is pre-test speed: 2.0 mm/s, test speed: 1.0 mm/s, post-test speed: 10.0 mm/s and distance: 8mm, time: 30 s, trigger type: auto 5 g. Accessory: AACC 36 mm cylinder probe with radius (P/36R) using 5 kg load cell.

2.2.12. Statistical Analysis

All experiments were performed in triplicate. The results were presented as mean ± S.D. and subject to Duncan test. P-values of less than 0.05 were considered statistically significant. The analysis was performed using SPSS statistics 22.

3. Results and Discussion

3.1. Proximate Composition of Fresh White Radish (Raphanus sativus L.) Roots

Proximate composition of fresh white radish roots is summarized in Table 1. The moisture content of white radish roots collected reached 96.1% and total dry mass content was 3.1% including 0.73% of protein and 2.67% of carbohydrate. Based on those results, the protein content and carbohydrate per dry matter in the material are calculated at 25.54% and 86.12%, respectively.

Table 1. Composition of the fresh white radish.

Composition	Moisture	Protein	Lipid	Carbohydrate	Total Ash
Content (%)	96.1 ± 1.35	0.73 ± 0.12	<0.1	2.67 ± 0.16	0.46 ± 0.27

3.2. Single Factor Analysis for the Extraction of WRE Using Ethanol as the Solvent

Figure 3 visualized total flavonoid content and Trolox equivalent antioxidant capacity (TEAC) of WRE with relation to different experimental parameters. It was indicated in the Figure 3 that both TEAC and TFC content peaked at the ethanol concentration of 75% and decreased thereafter. In the ethanol concentration range from 0 to 75%, the increasing trend of TFC could be attributed to the improved mass diffusivity of the materials to the media. To be specific, due to the higher polarity of the ethanol in comparison to the water, hydrogen-bonding of flavonoids with ethanol is better promoted by ethanol molecules [25]. This leads to improved dragging of flavonoids into media. On the other hands, excessively high ethanol concentration causes the solution to evaporate rapidly over time, overturning the said diffusivity improvement and in turn lowering the TFC.

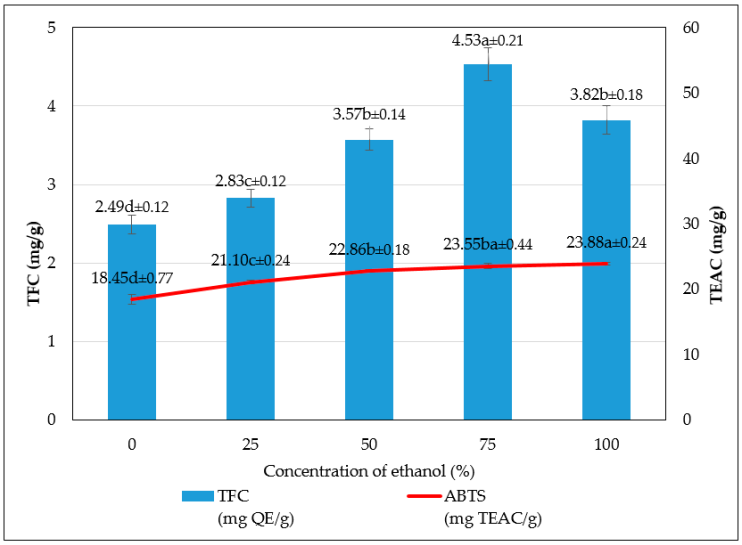

Figure 3. The effect of ethanol concentration on the total flavonoid content (TFC) and Trolox equivalent antioxidant capacity (TEAC) on WRE.

In the results of the next experiment (Figure 4), conducted at ethanol concentration 75%, it was found that at the material/solvent ratio of 1:50, the TEAC and TFC peaked at 25.1 and 5.2 mg/g, respectively. However, closer examinations of test statistics and the data revealed that these peak figures were statistically indifferent to those observed at the ratio of 1:30. In addition, the process performed at the high material/solvent ratio showed no economic advantages. The explanation for the relationship could be three-fold. First, the phenomenon could be due to the enlarged contact area between the materials and the extraction solvent, leading to increased extraction efficiency. Second,

the gradient in concentration between solid and the solvent might have accelerated the mass transfer, improving the flavonoid yield [26]. Third, the material/solvent ratio could alter the equilibrium constant of the reaction and the recovery efficiency, however only to a certain threshold where the rate of yield starts to diminish with increasing ratio [27].

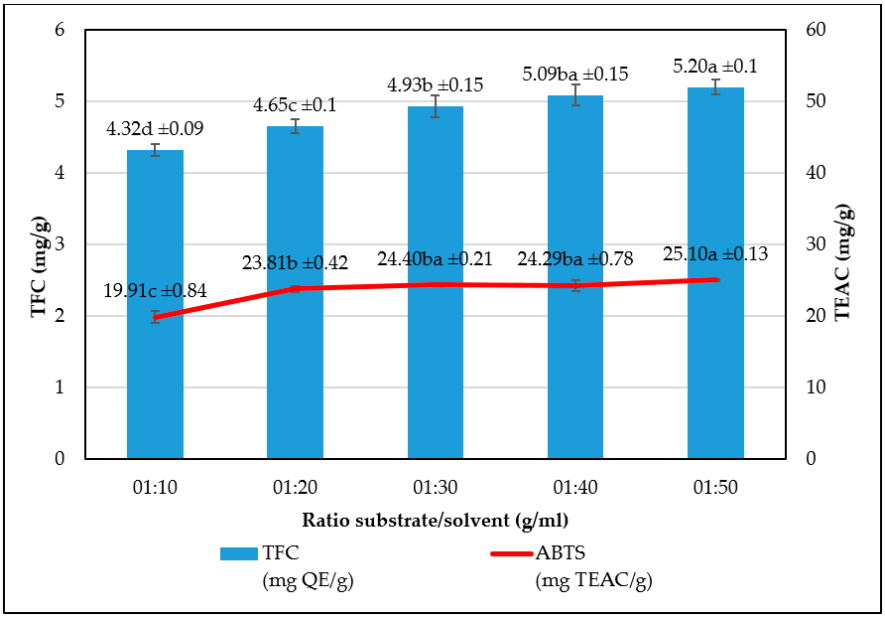

Figure 4. The effect of ratio substrate/solvent to extraction process.

The effect of temperature on the flavonoid yield and antioxidant activity of the WRE was described in the Figure 5. Experiments were performed at ethanol concentration of 75% and material/solvent ratio of 1:30. As the temperature rose from 45 to 50 °C, both TEAC and TFC witnessed a sharp rise to highest levels, at 28.26 and 6.25 mg/g, respectively. Afterwards, elevating the temperature seemed to gradually reduce both indicators. Since the high temperature could reduce viscosity of the solution, physical processes that involve diffusive and convective transport of nutrients could be facilitated. Furthermore, elevated temperature of the solution may play an important part in breaking down plant cell walls and linkages inside the material such as protein–phenol or phenol–polysaccharide links. Both explanations lead to increased TEAC and flavonoid content. However, excessively high temperature could adversely affect the flavonoid and other nutrients through decomposition or combination with others component, partially contributing to their change in oxidation properties.

Extraction time and its corresponding flavonoid yield and TEAC is shown in the Figure 6. At duration of 3 and 3.5 h, the highest level in TFC and TEAC was achieved. However, 3 h was selected as this duration is economical. In addition, yields obtained at prolonged periods, such as 3.5 and 4 h, showed no clear difference in comparison to yield at 3 h. The threshold could be explained by the Fick's second law of diffusion asserting that the equilibrium concentration of solute can be only achieved after a short period of time and short extraction time will not guarantee the extraction efficiency of antioxidant compounds [28]. Therefore, the final set of experimental parameters consisted of extraction temperature of 50 °C, the time of 3 h, the ratio 1:30 and ethanol concentration of 75%. These conditions correspond to the TFC content of 6.25 ± 0.1 (mg/g) and TEAC of 28.95 ± 0.43 (mg/g).

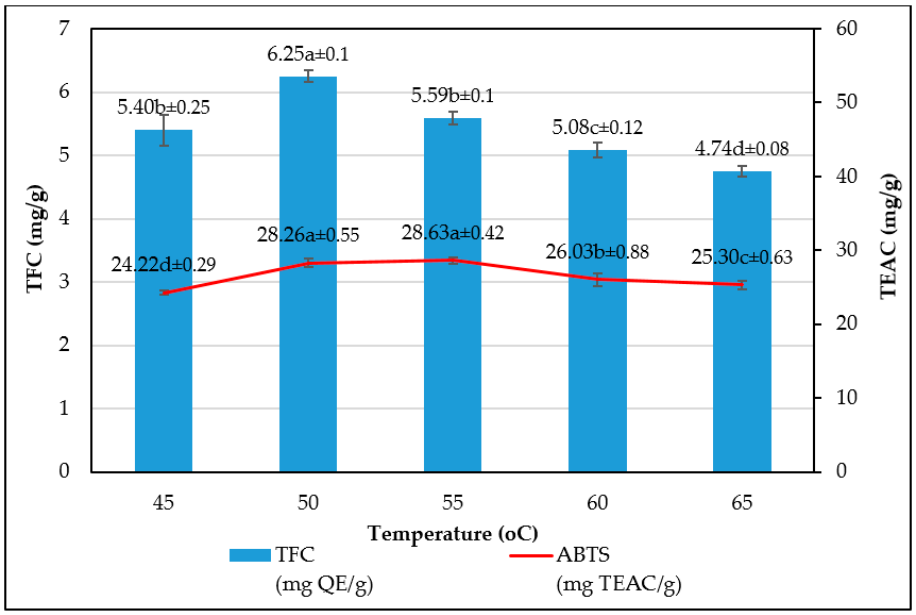

Figure 5. The effect of temperature to extraction process.

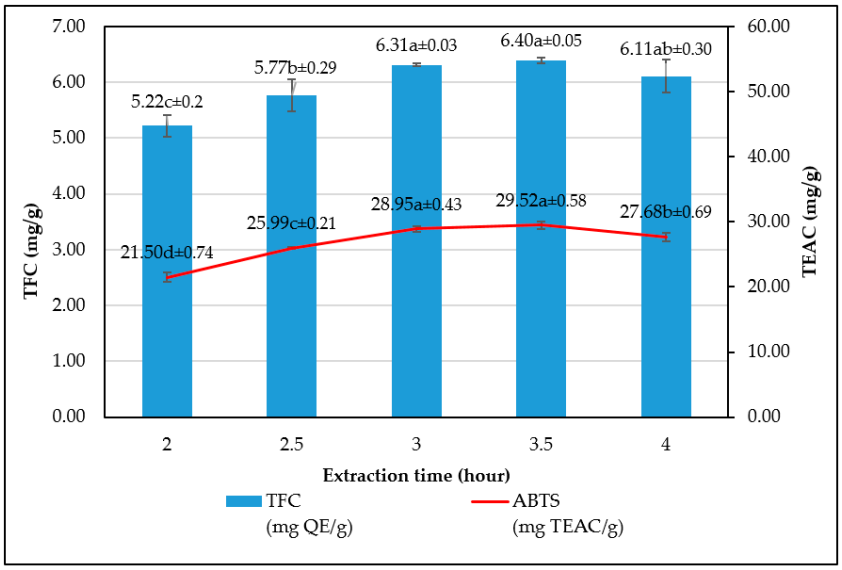

Figure 6. The effect of extraction time to extraction process.

3.3. Antifungal Susceptibility Testing by Disk Diffusion

Figure 7 shows the inhibition zones of various wells filled with WRE at different concentrations and with the positive control of 0.2 mg/mL natamycin. It was shown that the antifungal capacity of WRE against the four fungus species was negligible at the low concentration of 25 mg/mL. At this concentration, among four species, the largest diameter was observed in the disc inoculated with *Aspergillus niger*, at d = 10.22 ± 1.17 mm. Among four selected WRE concentrations, the concentration of 75 mg/mL showed the highest inhibitory effect against four species, of which the largest zone is observed against *Aspergillus niger* with d = 19.55 ± 1.68 mm. Apparently, the WRE ineffectively inhibited the growth of *Aspergillus flavus*, as demonstrated by very low diameters of well 1 to 5 in the *Aspergillus flavus* disc. In summary, we found that WRE exhibited fungicidal activities against most of the fungal species, especially *Aspergillus niger*, and that the WRE concentration of 75 mg/mL showed superior inhibitory effects to other concentrations and even to the positive control.

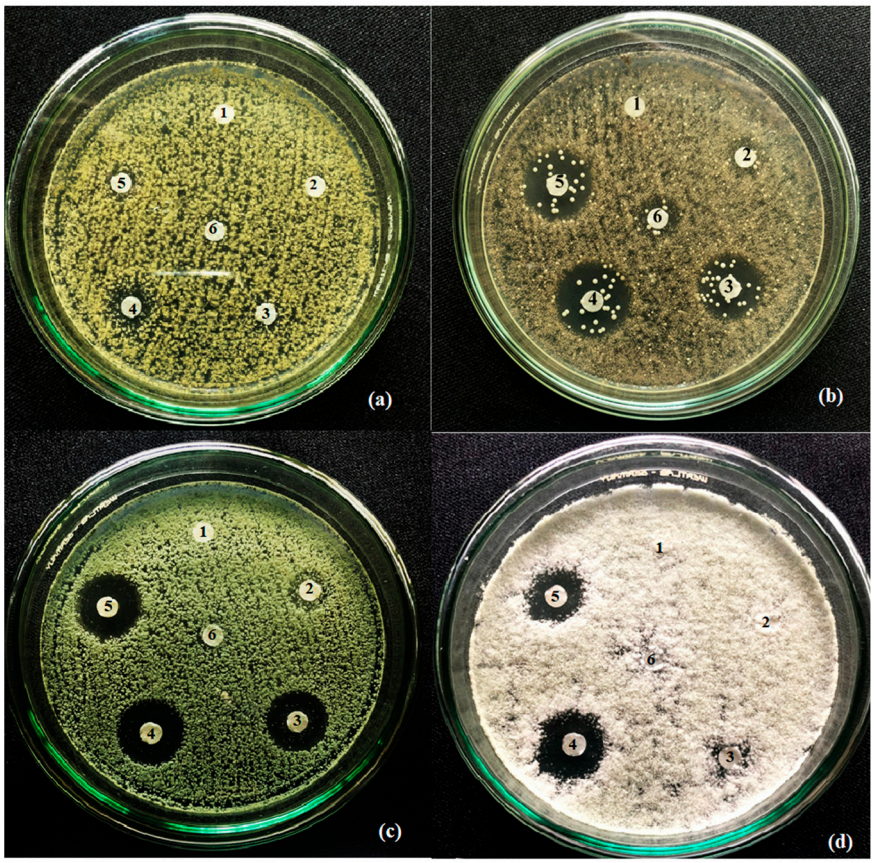

Figure 7. Antifungal susceptibility testing against (**a**) *Aspergillus flavus* NBRC 33021; (**b**) *Aspergillus niger* NBRC 4066; (**c**) *Aspergillus clavatus* NBRC 33020; (**d**) *Fusarium solani* NBRC 31094; (1): Negative control—DMSO 5%; (2): WRE 25 mg/mL; (3): WRE 50 mg/mL; (4): WRE concentrate 75 mg/mL; (5): WRE 100 mg/mL; (6): Positive control—natamycin 0.2 mg/mL.

3.4. Minimal Fungicidal Concentration (MFC)

Minimal fungicidal concentration (MFC) is defined as the lowest concentration of an antimicrobial agent that causes a specified reduction the visible growth of a microorganism in an agar or broth dilution susceptibility test. To determine MFC, various WRE concentrations of lower than 75 mg/mL were investigated against four fungal species and the results were compared with those of the natamycin in separate discs. The comparison is shown in Figure 8, showing a positively proportional correlation between concentration and inhibition. Table 2 details the growth of fungal colonies with respect to WRE and natamycin concentration. It was observed that at the WRE concentration of 30 mg/mL, all four species were inhibited and *Aspergillus niger* was the most susceptible species to antifungal agents, followed by *Aspergillus clavatus*. The minimum MFC value of natamycin was recorded at 0.2 mg/mL for *Aspergillus niger* (Table 3). In general, the lowest natamycin concentration where no growth was observed after 72 h of incubation was lower than that of WRE concentration. Our result was similar to the recent findings of Salvosa et al. who reported that *Aspergillus flavus* showed no growth when treated with natamycin with concentrations ranging from 150–40000 µg/mL, which was higher than MFC for *Fusarium solani* (10 µg/mL) [29].

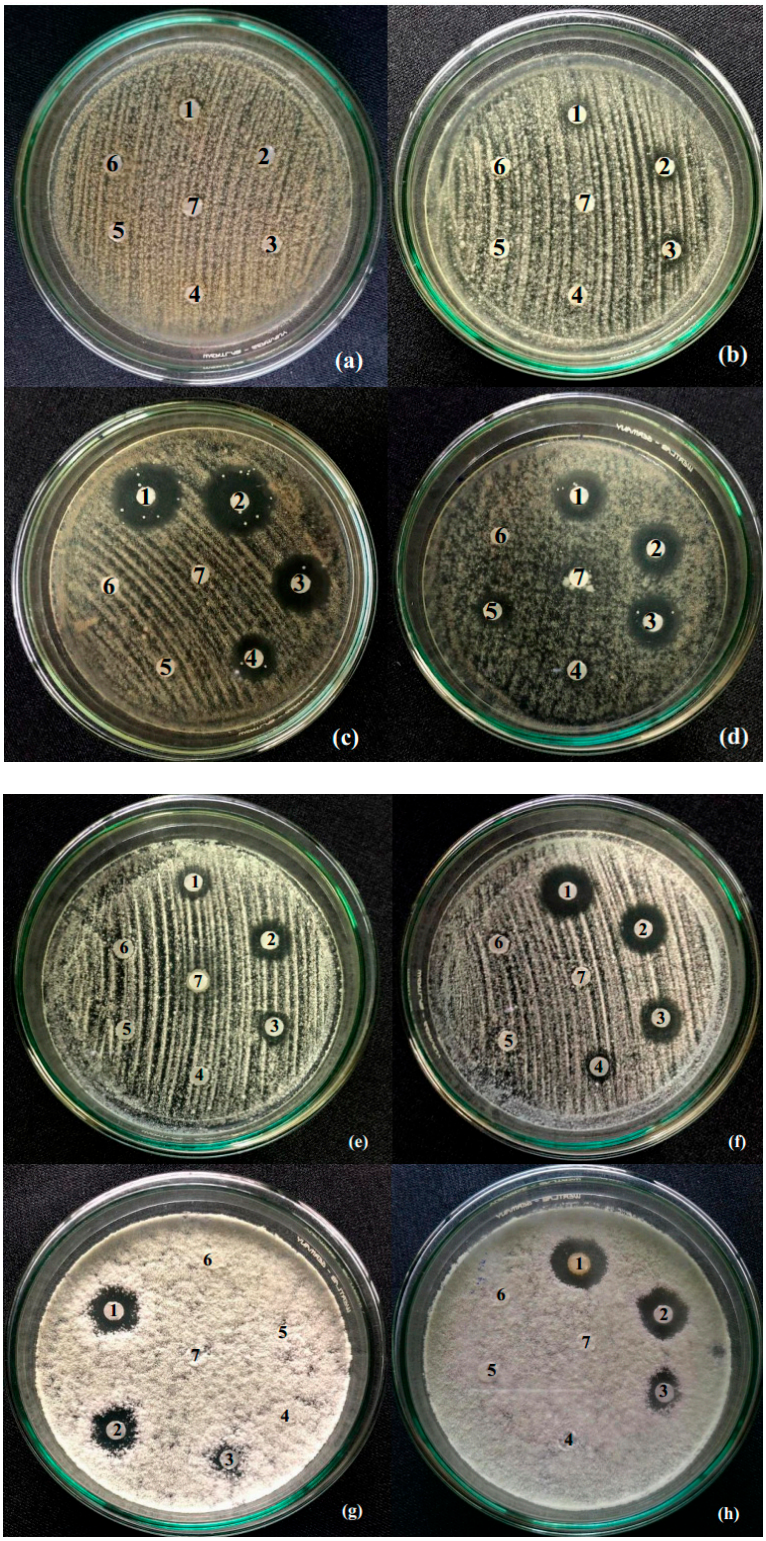

Figure 8. Minimum fungal concentration (MFC) of (**a**,**b**): *Aspergillus flavus* NBRC 33021; (**c**+**d**): *Aspergillus niger* NBRC 4066; (**e**+**f**): *Aspergillus clavatus* NBRC 33020; (**g**+**h**): *Fusarium solani* NBRC 31094; disk (**a**,**c**,**e**,**g**): Natamycin and disk (**b**,**d**,**f**,**h**): WRE.

Table 2. Inhibition zone diameter (mm) for molds of white radish extract (WRE) (*).

Concentration	Inhibition Zone Diameter (mm)			
	Aspergillus flavus **NBRC 33021**	***Aspergillus niger*** **NBRC 4066**	***Aspergillus clavatus*** **NBRC 33020**	***Fusarium solani*** **NBRC 31094**
DMSO 5% (*v*/*v*)	NI	NI	NI	NI
WRE 25 mg/mL	NI	$10.22^{b} \pm 1.17$	$8.22^{b} \pm 0.39$	$6.00^{a} \pm 0.00$
WRE 50 mg/mL	$6.22^{a} \pm 0.39$	$17.89^{bc} \pm 1.39$	$15.34^{c} \pm 0.58$	$6.61^{a} \pm 0.25$
WRE 75 mg/mL	$9.11^{b} \pm 1.5$	$19.55^{c} \pm 1.68$	$17.72^{c} \pm 0.25$	$17.50^{b} \pm 0.73$
WRE 100 mg/mL	$7.00^{a} \pm 0.67$	$19.11^{c} \pm 0.77$	$17.66^{c} \pm 0.58$	$14.83^{b} \pm 0.60$
Natamycin 0.2 mg/mL	$6.33^{a} \pm 0.58$	$11.5^{b} \pm 1.02$	$6.22^{a} \pm 0.39$	$6.00^{a} \pm 0.00$

(*) mean value ± SD of triplicate experiments, $n = 3$ (the zone of inhibition mm including a disk of 6 mm in diameter). NI = no inhibition.

Table 3. MFC values of WRE and natamycin against the four species.

Species	Concentration of WRE (mg/mL)							Concentration of Natamycin (mg/mL)						
	50	40	30	20	10	5	2.5	0.8	0.6	0.4	0.2	0.1	0.05	0.025
Aspergillus flavus NBRC 33021	+	+	+	-	-	-	-	+	+	+	-	-	-	-
Aspergillus niger NBRC 4066	+	+	+	+	+	-	-	+	+	+	+	-	-	-
Aspergillus clavatus NBRC 33020	+	+	+	+	-	-	-	+	+	+	-	-	-	-
Fusarium solani NBRC 31094	+	+	+	-	-	-	-	+	+	+	-	-	-	-

(+) No growth, (-) Growth.

3.5. Shelf-Life Study of Sponge Cake in Air-Cooled Condition 20 °C

3.5.1. Water Activity

Water activity, unlike water content, can determine shelf stability of foods since the development potential of spoilage-causing microorganisms could be predicted based on the water activity. The water activity of a food is also instrumental in maintaining its chemical stability [30]. Therefore, food products can be safely produced and preserved by lowering water activity to the point that microorganisms cannot grow. Figure 9 illustrates water activity measurements (Aw) of sponge cake added with WRE and sponge cake added with potassium sorbate with relation to different storage periods at 20 °C. Overall, both cake samples exhibited similar water activity ranging from 0.65–0.71 and the trend was gradually increasing over the examined course.

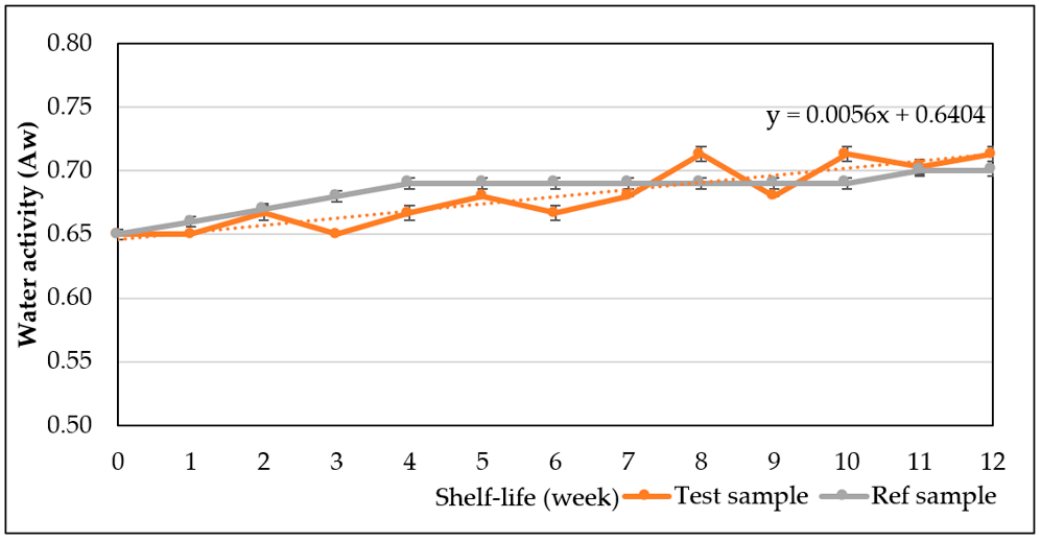

Figure 9. Water activity at 20 °C after various storage periods.

3.5.2. Sensory Evaluation

Overall, the sensory evaluation (SE) score of panelists gradually decreased from 6, evaluated with initial cakes, to 3.08, evaluated at cakes after 12 weeks of storage (Figure 10). These figures indicated that the quality of sponge cake added with WRE was acceptable even after 12 weeks of storage under 20 °C.

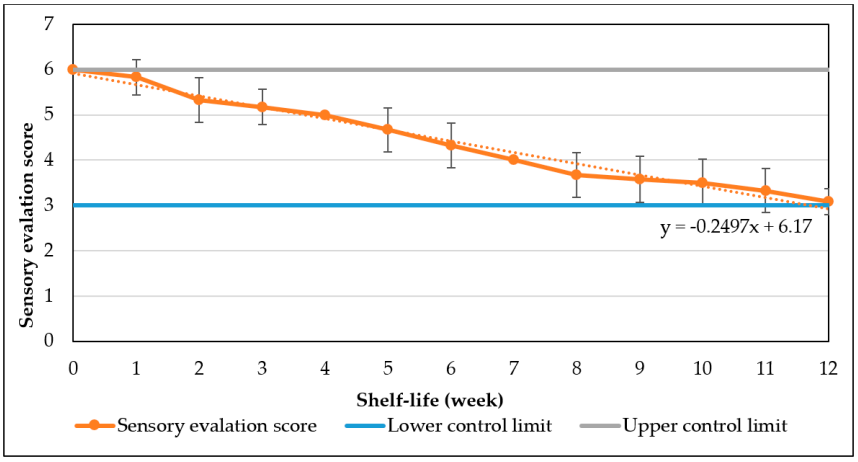

Figure 10. Sensory evaluation score in air-cooled condition 20 °C after various storage times.

3.5.3. Texture Analysis

Firmness of sponge cakes after various storage times are visualized on Figure 11 and in Table 4. Maximum firmness of sponge cake was reached at the 11th week (732.08 ± 3.37). In cakes after 12 weeks of storage, the texture was slightly softer (578.15 ± 0.07). In comparison with the standard sample (sponge cake stored at 5 °C), the surface of the cake was drier. The explanation could be due to the natural starch degradation occurring in the sample. For this reason, it was suggested that application of WRE 30 mg/mL did not improve texture of sponge cake.

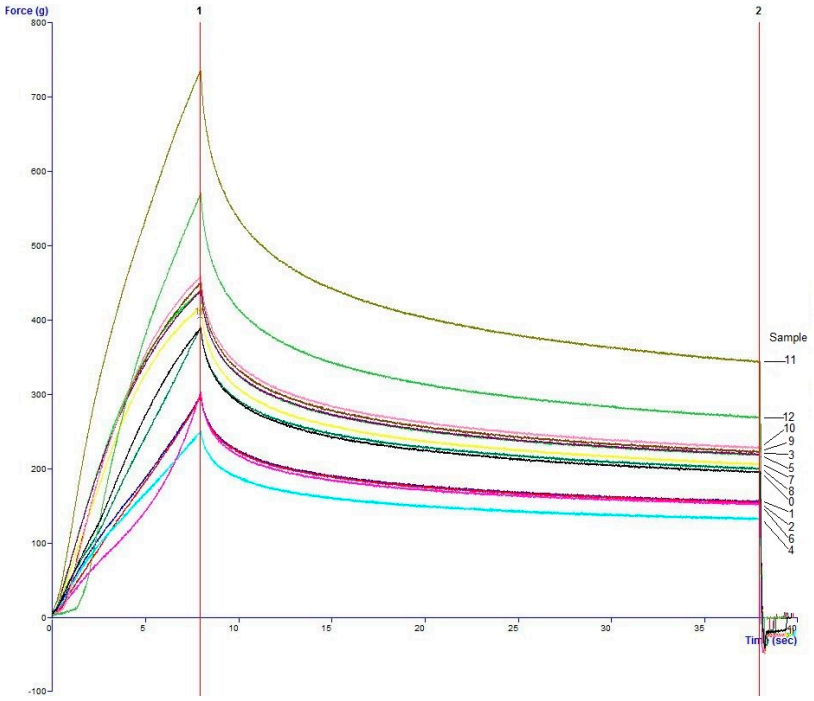

Figure 11. Firmness of sponge cakes in air-cooled condition 20 °C and after various storage times.

Table 4. Firmness of sponge cakes in air-cooled condition.

Time (Week)	0	1	2	3	4	5	6
Average Firmness (F)	370.23 ^{a} ± 15.03	450.06 ^{b} ± 30.74	419.96 ^{b} ± 19.49	355.64 ^{a} ± 10.80	312.23 ^{c} ± 19.49	445.19 ^{b} ± 4.24	295.36 ^{c} ± 26.79
Time (Week)	**7**	**8**	**9**	**10**	**11**	**12**	
Average Firmness (F)	335.39 ^{a} ± 8.72	394.70 ^{a} ± 33.42	455.97 ^{b} ± 19.34	462.47 ^{b} ± 2.14	732.08 ^{d} ± 3.37	578.15 ^{e} ± 0.07	

Means with different superscripts in the same column are significantly different at $p < 0.05$ by Duncan's multiple range test.

3.5.4. Microbiological Indication

Sponge cake added WRE was evaluated for various microbiological indicators after various storage periods. The results are presented in the Table 5.

Table 5. Microbiological testing results of sponge cake after storage time.

Microorganisms	Unit	Permissible Range (*)	4th Week	8th Week	12nd Week	12nd Week (Control)	Method (**)
Bacillus cereus	Cfu/g	<10	ND	ND	ND	ND	AOAC 980.31
Clostridium perfringens	Cfu/g	<10	ND	ND	ND	ND	ISO 7937:2004
Coliforms	Cfu/g	<10	ND	ND	ND	ND	ISO 4832:2006
E. coli	Cfu/g	3	ND	ND	ND	ND	ISO 16649-2:2001
Staphylococcus aureus	Cfu/g	<10	ND	ND	ND	ND	ISO 6888-1:2003
Molds and yeasts	Cfu/g	10^2	10^2	10^3	2×10^3	10	ISO 21527-2:2008
Total aerobic microbial count	Cfu/g	10^4	10^4	2×10^4	3×10^4	10^2	ISO 4833-1:1013

(*) In accordance to the decision No.46/2007/QD-BYT of the Ministry of Health, Vietnam on promulgation regulation of maximum level of biological and chemical pollution in food. (**) LOD of method = 10 cfu/g. ND—Not detected. The control was sponge cake that added with potassium sorbate and stored at 20 °C for 11 weeks.

After 8 weeks stored at air-cooled condition 20 °C, the sponge cake added with WRE showed higher total aerobic microbial count, molds and yeasts than the standard requirement. Based on the results, the storage time of sponge cake added with WRE is approximately 8 weeks at air-cooled condition of 20 °C.

3.6. Shelf-Life Study of Sponge Cake in Accelerated Condition 37 °C

3.6.1. Water Activity

Figure 12 shows the range of water activity of sponge cake at different storage periods. Overall, water activity ranged from 0.65 to 0.71, demonstrating that sponge cake added with WRE can inhibit the activity of most bacteria and yeasts. In this temperature condition, one additional week of storage rises the water activity of the sponge cake by approximately 0.016.

3.6.2. Sensory Evaluation

Under 37 °C, it was indicated that the sensory score fell rapidly and approximated the lower control limit of 3.00 for cake samples after 4 weeks of storage (Figure 13). This number is lower in comparison with the previous sensory results where the lower limit was reached at cake samples stored after 12 weeks. After four weeks, SE score was 3.25 ± 0.45 (>3, lower control limit), suggesting acceptable quality of the cake.

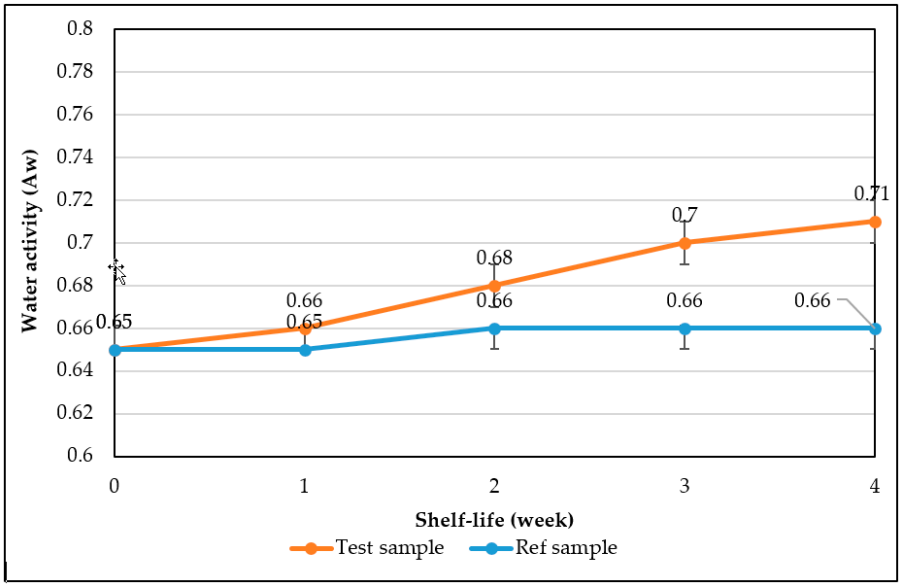

Figure 12. Water activity of sponge cake in accelerated condition 20 °C after various storage times.

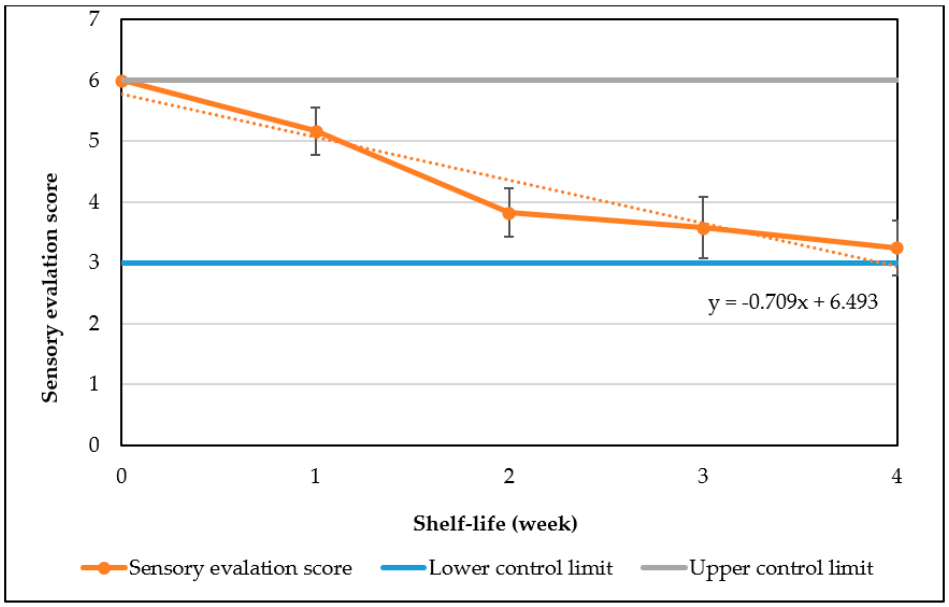

Figure 13. Sensory evaluation score in accelerated condition 37 °C.

3.6.3. Texture Analysis

The results for the firmness of sponge cake stored under 37 °C are shown in the Table 6 and Figure 14. The firmness of sponge cake increased rapidly and reached the highest value at the third week (792.53 F) which is equivalent to the values of cakes stored in air-cooled condition after 11 weeks in the previous experiment. The accelerated texture degradation could be partially attribute to the elevated temperature [31].

Table 6. Firmness of sponge cakes in accelerated condition 37 °C.

Time (week)	0	1	2	3	4
Average Firmness (F)	371.89 ^{a} ± 13.67	578.34 ^{b} ± 1.73	525.37 ^{b} ± 1.98	792.53 ^{c} ± 1.30	667.56 ^{d} ± 1.05

Means with different superscripts in the same column are significantly different at $p < 0.05$ by Duncan's multiple range test.

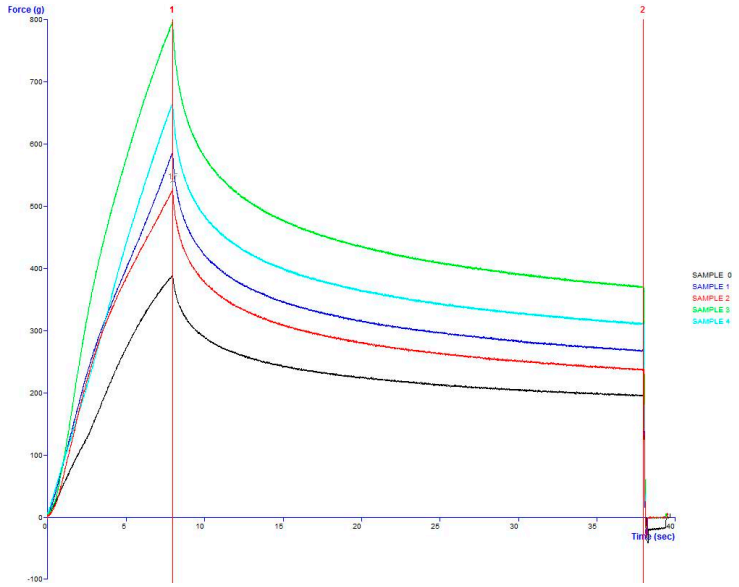

Figure 14. Firmness of sponge cakes in accelerated condition 37 °C.

3.6.4. Microbial Testing

Sponge cake added with WRE and stored in at 37 °C was tested for microbial indicators. The results are shown in Table 7.

Table 7. Microbial testing results of sponge cake after different storage periods at 37 °C.

Microorganisms	Unit	Permissible Range (*)	4th Week	8th Week	12nd Week	12nd Week (Control)	Method (**)
Bacillus cereus	Cfu/g	<10	ND	ND	ND	ND	AOAC 980.31
Clostridium perfringens	Cfu/g	<10	ND	ND	ND	ND	ISO 7937:2004
Coliforms	Cfu/g	<10	ND	ND	ND	ND	ISO 4832:2006
E. coli	Cfu/g	3	ND	ND	ND	ND	ISO 16649-2:2001
Staphylococcus aureus	Cfu/g	<10	ND	ND	ND	ND	ISO 6888-1:2003
Molds and yeasts	Cfu/g	10^2	ND	2×10^3	10^4	10	ISO 21527-2:2008
Total aerobic microbial count	Cfu/g	10^4	10^3	3×10^4	4×10^4	10^2	ISO 4833-1:1013

(*) In accordance with decision No.46/2007/QD-BYT of the Ministry of Health, Vietnam on promulgation regulation of maximum level of biological and chemical pollution in food. (**) LOD of method = 10 cfu/g. ND—Not detected. The control was sponge cake that added with potassium sorbate and stored at 37 °C for 11 weeks.

After three weeks of storage at 37 °C, the WRE-added sponge cake still maintained most of microorganism indicators below the permissible range. However, at the 8th week, sponge cake added with WRE exceeded the permissible upper limit in terms of total number of yeasts and molds as well as the total number of aerobic microorganisms. For comparison, the control cake, which was added with potassium sorbate, exhibited excellent anti-microorganism activity with all indicators below the permissible range even after 11 weeks. Therefore, it is suggested that the WRE could only act as a anti-microorganism agent of moderate activity and sponge cake added with WRE should have the shelf-life of under 4 weeks, most preferably 2 weeks, when stored at 37 °C.

4. Conclusions

The study has optimized the total flavonoid content and antioxidant activity of the white radish root extract with regard to four extraction parameters including concentration of ethanol, ratio of solvent/material, temperature and time. Optimized parameters consisted of extraction temperature of 50 °C, the time of 3 h, ratio of 1:30 and ethanol concentration of 75%. These conditions correspond to the TFC content of 6.25 ± 0.1 mg/g and TEAC of 28.95 ± 0.43 mg/g. In vitro disk diffusion assay showed

that WRE could inhibit four fungal species of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus clavatus*, and *Fusarim solani* with concentration of 75 mg/mL. For *Aspergillus flavus* and *Fusarim solani*, the MIC of WRE was 30 mg/mL. The figures were 20 and 10 mg/mL for *Aspergillus clavatus* and *Aspergillus niger*, respectively. To maintain acceptable physical, sensory and microbial parameters, sponge cakes added with WRE are suggested to have the shelf-life of 8 weeks at storage temperature of 20 °C or 2 weeks at 37 °C. These results suggest the use of white radish as a potential material for manufacture of natural preservatives.

Author Contributions: Investigation, H.H.D., P.T.K.N., L.T.H.A. and V.T.T., Supervision, D.T.A.D.; Writing—original draft, H.H.D.; Writing—review & editing, D.C.N.

Funding: This research received no external funding.

Acknowledgments: We are thankful to the researchers from Technology Laboratory of Faculty of Chemical Engineering, HCMC University of Technology, Vietnam National University, for their support and for providing valuable advice during this work.

Conflicts of Interest: The authors declare no conflict of interest.

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