Effect of Solid-State Fermentation (SSF) on the Antioxidant and Hypoglycemic Activities of Jackfruit (*Artocarpus Heterophyllus* Lam.) By-Products using *Aspergillus Niger*, *Aspergillus Oryzae* and *Rhizopus Oryzae*

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Abstract

The objective of this experiment was to study the effect of Solid-State Fermentation (SSF) on jackfruit by-products using filamentous fungi. Rags from a ripen jackfruit were fermented for 5, 10 and 15 days by *Aspergillus niger*, *Aspergillus oryzae* and *Rhizopus oryzae* at 30 °C. Then the bioactive compounds were extracted using methanol. Total phenolic content, total flavonoid content, antioxidant activity and hypoglycemic activity of each liquid extract were estimated. The results showed significant increase and improvement in the samples in which SSF was applied. The highest total phenolic contents were found in fermented products with *A. niger* and *R. oryzae* with an average of 14.16 mg GAE/g D.S which was almost 3 times over the concentration found in unfermented products (4.83 mg GAE/g D.S). The same fermented products showed a significant increase in total flavonoid content which was 2 times higher than unfermented products with an average of 31.79 mg QuE/g D.S for fermented products and 15.08 for unfermented. The antioxidant activity was correlated to the total phenolic and flavonoid contents with the highest activity of around 76 % found in fermented products against 55 % for the unfermented ones. Finally, hypoglycemic activity was also found to be higher in fermented products than the unfermented with the best activity obtained in 5-days fermented product with *A. oryzae* (35.62 %) which was 8 times higher than non-fermented products (4.52 %). The present study showed that Solid-State Fermentation has a great potentiality to improve and enhance antioxidant and hypoglycemic activities of plant by-products.

Keywords: Jackfruit rags, Solid-state fermentation, Filamentous fungi, Antioxidant activity, Hypoglycemic activity

Introduction

Cancer and diabetes mellitus are some of the most serious health issues in this 21st century. According to estimates from the International Agency for Research on Cancer (IARC), in 2018 there were 17.0 million new cancer cases and 9.5 million cancer deaths worldwide. By 2040, the global burden is expected to grow to 27.5 million new cancer cases and 16.3 million cancer deaths [1]. On the other hand, according to a study done in 2020, an estimated 4.2 million deaths among 20 - 79-year-old adults were attributed to diabetes mellitus, a metabolize disease characterized by high blood glucose level. It was assessed to be 11.3 % of global deaths [2]. Scientific research about cancer and diabetes is then of great interest for the world. Not only therapeutic compounds should be on focus, but preventive products also need to be found as the saying goes it is better to prevent than to cure. Lot of research have already been carried out. Among them, a growing interest in plant-based natural compounds is manifest. These bioactive compounds produced by plants are usually secondary metabolites which can be categorized into different classes: tannins, phenolic acids, flavonoids, terpenoids, alkaloids and steroids [3]. They are characterized by great structural diversity which offers them the advantage of greater variation in biological activity [4]. Found in many plants, phenolic acids and flavonoids are well known for their antioxidant property, making them great potential anticancer agents [5]. Hypoglycemic property, antimicrobial activities, anti-inflammatory properties, and many other therapeutic virtues were also reported [6,7]. Interestingly, unlike synthetic drugs which have serious undesirable adverse effects, plant-based therapeutics have been proven effective, non-toxic, and inexpensive [8]. Hence, their acceptability is significantly increasing all over the world. Currently, they constitute a hotspot in the area of cosmetic, pharmaceutical and natural food supplement markets [7].

Among the multiple medicinal plants known in Thailand, jackfruit (*Artocarpus heterophyllus* Lam) or Khanoon in Thai, the largest tree born fruit in the world, is found to be very rich in medicinal properties among which antioxidant and hypoglycemic activities are particularly high. Several studies have revealed that its leaf, root, bark, and fresh fruit contain various bioactive compounds such as flavonoids, phenolic acids, organic acids, carotenoids, etc. These bioactive compounds confer to jackfruit high antioxidant and hypoglycemic activities [9]. A study on the different parts of jackfruit showed that the peel, about 46 % of the fruit, usually underutilized and mainly discarded as waste or fertilizer, had higher total phenolic and flavonoid contents, and higher antioxidant activity than the pulp, the flake, and the seed [7]. The same study also revealed a higher enzyme α-glucosidase inhibitory activity in the peel. Other research carried out by Suchithra & Subramanian, were interested in the jackfruit rags, the whitish yellow filament like structures present in the fruit. Their results showed that the rags are non-toxic and possess significant antidiabetic and antioxidant properties [10]. Thus, the overall jackfruit by-products could have high potentiality of becoming privileged sources in preventing and reducing the risk of cancer and diabetes. More interestingly, the antioxidant and hypoglycemic properties of these underutilized by-products could be improved through Solid State Fermentation (SSF) using fungi.

The ability of SSF to improve antioxidant and hypoglycemic properties of plant materials has been widely demonstrated in the literature. It increases ferulic acid and DPPH scavenging activity. It also enhances the inhibition of α-glucosidase, peroxidase, and polyphenol oxidase [11]. The most used microorganisms for this purpose are filamentous fungi such as *Aspergillus niger*, *Aspergillus oryzae* and *Rhizopus oryzae* which have been proven to have great potential to produce bioactive compounds by SSF [12-15]. In our acknowledgement, the effect of this promising SSF on the antioxidant and hypoglycemic activities on jackfruit by-products has not been investigated yet. Meanwhile, the success of such an investigation on these permanent, abundant, and underutilized raw materials could result in a significant cost-effective contribution to the world's battles against cancer and diabetes.

In this work, jackfruit rags were fermented for different length of time using 3 different fungi: *Aspergillus niger*, *Aspergillus oryzae* and *Rhizopus oryzae*. Then, the polyphenol and flavonoid content, and the antioxidant and hypoglycemic activities of each sample were evaluated in comparison with the unfermented rags. The antioxidant activity was investigated by DPPH radical scavenging ability assay which is widely used in plant biochemistry to evaluate the properties of their constituents for scavenging free radicals [16]. The hypoglycemic activity was studied through the inhibition of the activity of α-glucosidase enzyme.

Materials and methods

Materials
A fresh ripe jackfruit was obtained from Suan Kaset Thippayasombat Co., Ltd. The rags were manually separated from the rest of the fruit and used for the experiments. Aspergillus niger (TISTR 3012), Aspergillus oryzae (TISTR 3014) and Rhizopus oryzae (TISTR 3024) were purchased from Thailand Institute of Scientific and Technological Research (TISTR). p-Nitrophenyl-α-D-glucopyranoside, α-glucosidase (yeast, EC 3.2.1.20), acarbose, 1,1-dipheny-1,2-picryl hydrazyl (DDPH), quercetin, gallic acid and Folin Ciocalteu reagent were acquired from Sigma Aldrich Co., Ltd. (St. Louis, MO). Methanol was purchased from Fisher (Fair Lawn, NJ).

Fermentation process
The fresh jackfruit rags (83.53 % w/w moisture content and pH 5.32) were chopped into small pieces. Then, 20 g were introduced in 250 mL Erlenmeyer flasks. After heat sterilization into an autoclave at 121 °C for 30 min, the substrate in each flask were inoculated with 1 mL of fungal spores suspended in sterilized water and containing an average of 6.3×10^5 spores/mL. The cultures were incubated at room temperature (30 °C) for various days (5; 10 and 15 days). The experiment was done with 3 replicates for each type of microorganism and each number of days.

Extraction of phytochemical compounds
The freshly fermented products were crushed into mortars using pestles and soaked in 90 % methanol at a solid/liquid ratio of 1:30 (g/mL). The extraction was then allowed to occur at room temperature for 24 h. Next, the extracts were obtained by filtering the mixtures using Whatman Filter paper Grade 4. Finally, the filtrates were evaporated and made to 50 mL with 90 % methanol to obtain the pure extracts.

Analysis of total phenolic content

The total phenolics of the extracts were determined using the Folin-Ciolcalteu reagent following the method described by Singleton and Rossi and Wiesneth with further modifications and adaptations [17-19]. The test sample (100 µL) was mixed with 500 µL of distilled water and 100 µL of Folin-Ciocalteu's phenol reagent. After 6 min, 1 mL of saturated sodium carbonate solution (7 % v/v in water) and 500 µL of water were added to the mixture. The reaction was kept in the dark for 90 min. After centrifugation at 4,000 rpm for 6 min, the absorbance of the different samples was measured at 760 nm wavelength. The phenolic content was calculated using a standard curve from gallic acid. The results were expressed in milligram Gallic Acid Equivalent per gram of Dry Sample (GAE/g D.S). All the measurements were carried out in triplicate.

Analysis of total flavonoid content

The total flavonoids were determined using the aluminum chloride method described in previous studies [20,21]. Quercetin was used to make the standard curve. Quercetin (5.0 mg) was dissolved in 100 mL methanol. Then, the standard solution was prepared by serial dilutions from 12.5 - 200 µg/mL using methanol. Next, 1.2 mL of diluted standard quercetin solutions or extract was mixed with 1.2 mL of 2 % aluminum chloride. After incubation for 60 min at room temperature, the absorbance of the solutions was measured at 420 nm wavelength. The total flavonoid concentration was calculated from the calibration curve. The results were expressed in mg Quercetin equivalent per g of Dry Sample (mg QuE/g D.S).

Determination of antioxidant activity by DPPH scavenging assay

The antioxidant activity was measured by DPPH scavenging ability following a modified method of Zhang *et al.* [6]. Each sample (50 µL) was mixed with 3 mL of 0.002 % freshly prepared DPPH in methanol. After incubation in darkness for 15 min at room temperature, absorbance was read against a blank using spectrophotometer at 517 nm wavelength. The scavenging ability was calculated as follows:

$$\text{Inhibition \%} = \frac{A-B}{A} \times 100 \qquad (1)$$

where A was the absorbance of control (without sample), and B was the absorbance of the sample. Gallic acid was used as positive reference.

Determination of α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity was determined according to the method of Kim *et al.* [22] and Nguyen *et al.* [23] with slight modifications. A 20 U/mL concentrated α-glucosidase (50 µL) in 0.01M phosphate buffer and 3mM p-nitrophenyl-α-D-glucopyranoside (50 µL) in 0.01 M phosphate buffer (pH 7) was added to the sample solution (2.2 mL) to start the reaction. The reaction was carried out at 37 °C for 30 min and stopped by adding 2 mL of 0.1 M Na_2CO_3. Then, the enzymatic activity was quantified by measuring the absorbance of the different solutions at 405 nm wavelength. Acarbose was used as reference.

The inhibitory ability was calculated as follows:

$$\text{Inhibition \%} = = \frac{Ac-(Ai-Aj)}{AC} \times 100 \qquad (2)$$

where Ac was the absorbance of control (without sample), Ai the absorbance of the test sample and Aj the absorbance of the negative control (without α-glucosidase).

Statistical Analysis

All samples were analyzed in triplicate and the results were presented as mean ± SD. The data obtained were statistically analyzed by R-program version R 2.15 at a level of $p < 0.05$ on factorial RCBD and Duncan's multiple range analysis. The variable "time of fermentation" was run as the block factor while the "type of filamentous fungi" used for the fermentation was the factor of interest.

Results and discussion

Determination of total phenolic and flavonoid compounds

Figure 1 below shows the total phenolic content in the samples of unfermented (control) and fermented jackfruit rags with different fungi and at different length of time. Results are explained in the unit of mg Gallic Acid Equivalent/g of Dry Sample (mg GAE/g D.S).
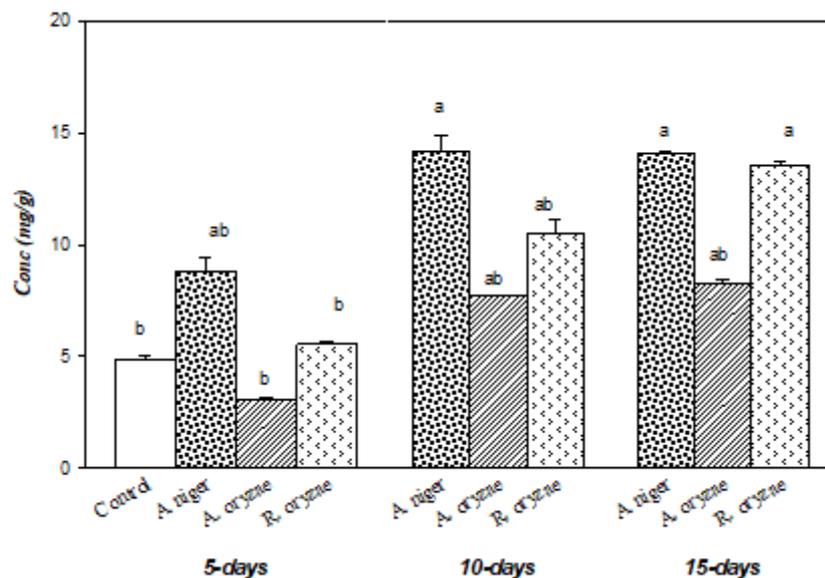
Figure 1 Total phenolic content of unfermented and fermented jackfruit rags at different time with *A. niger, A. oryzae* and *R. oryzae*.

Among all these samples, fermented products gave higher phenolic content than unfermented samples. Overall, total phenolic content kept increasing with the increase of fermentation time. A significant difference is noticeable between the total phenolic content of unfermented and fermented products. The highest total phenolic content was found after 10 days fermentation with A. *niger* (14.16 mg GAE/g D.S.) which was almost 3 times over the concentration found in non-fermented products (4.83 mg GAE/g D.S.). This concentration remained insignificantly different after 15 days ($p > 0.05$). The same level was reached with *R. oryzae* but after 15 days fermentation. *A. oryzae* showed lower phenolic compound overall even though a steady increase was noticed as time of fermentation was increasing. Therefore, *A. niger* appears to be the best fungi for phenolic compounds production using jackfruit rags as substrate. The optimum fermentation time using this specific microorganism is 10 days.

On the other hand, **Figure 2** below shows the total flavonoid content in the samples of unfermented (control) and fermented jackfruit rags with different fungi and at different lengths of time. Results are expressed Quercetin Equivalent per mL of Dry Sample (mg QuE/g D.S).
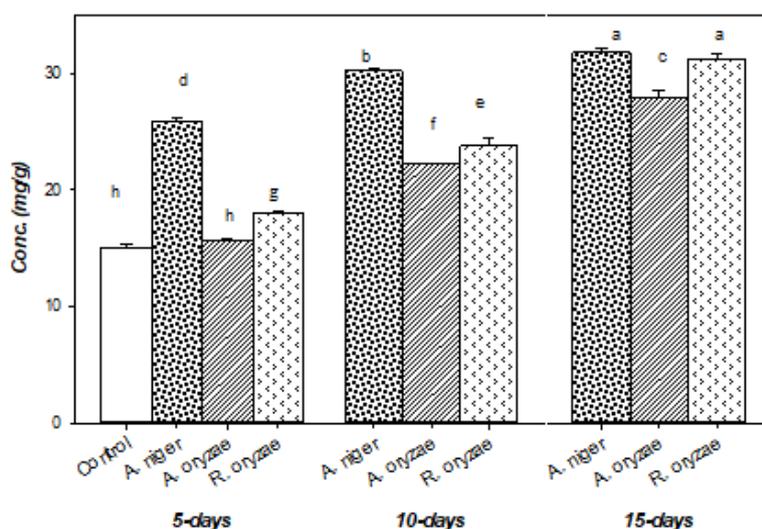

Figure 2 Total flavonoid content of unfermented and fermented jackfruit rags at different time with *A. niger, A. oryzae* and *R. oryzae*.

These results also demonstrated that fermented jackfruit rags had higher flavonoid content than the unfermented ones. Unfermented rags gave the lowest flavonoid content followed by 5-days fermentation with *A oryzae* respectively at 15.08 and 15.66 mg QuE/g D.S. It can also be clearly seen that the flavonoid content of the fermented products increased over time as the number of days of fermentation was increasing. The highest content was found in 15-days fermented products with *A. niger* (31.79 mg QuE/g D.S). The second one was obtained in 15-days fermented products with *R. oryzae* (31.18 mg QuE/g D.S). Hence, *A. niger* appeared to be the best microorganism for flavonoid production at the optimum fermentation time of 15 days.

All the above results showing a significant increase of phenolic and flavonoid contents of jackfruit rags by SSF using *A. niger*, *A. oryzae and R. oryzae* comply with that of previous reports [12-14,24]. They clearly showed the ability of these filamentous fungi to increase phenolic and flavonoid compounds in plants materials. The mechanism underlying this process would be the production of different hydrolytic enzymes such as α-amylase and protease which can degrade the carbohydrate linkages of the plant materials and release their phenolic and flavonoid compounds [24]. As obtained in our experiment, Magro *et al.* [25] also found that *A. niger* had more effect on the phenolic and flavonoid content than *A. oryzae*. In the same study, the length of fermentation was also lower for *A. niger* (48 h). Likewise, other previous studies revealed a high effect of *R. oryzae* on phenolic content [26].

Determination of antioxidant activity by DPPH scavenging assay
Figure 3 below shows the antioxidant activity of extracts from fermented and unfermented jackfruit rags. The results are explained in the unit of percentage of inhibition.
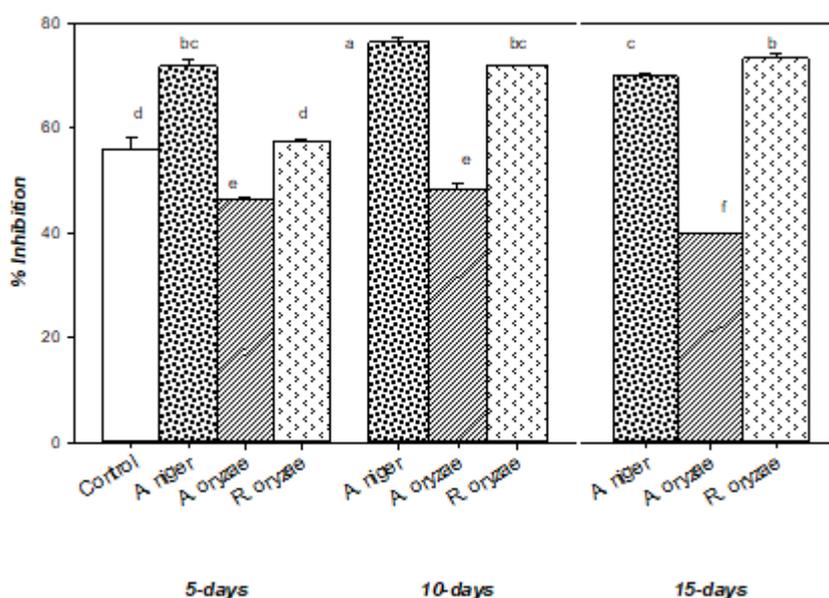

Figure 3 Antioxidant activity of unfermented and fermented jackfruit rags at different time with *A. niger, A. oryzae* and *R. oryzae*.

From these results, it can be clearly seen that the antioxidant activities in fermented products were higher than unfermented ones except the products obtained from the fermentation with *A. oryzae* which gave lower activity than the unfermented. The highest antioxidant activity was found in 10-days fermented products with *A. niger* (76.43 %). This result was closely followed by 15-days fermented product with *R. oryzae* (73.31 %).

This ability of SSF to increase antioxidant activity of plant materials was also reported in previous research [12-14,24]. Besides, a comparison between the antioxidant activity and the total phenolic and flavonoid content obtained in this experiment showed that there is a correlation among them. The samples with the highest phenolic and flavonoid contents revealed in **Figures 1** and **2** (fermented products with *A. niger* and *R. oryzae*) showed the highest antioxidant activities in **Figure 3** while the sample with lowest phenolic and flavonoid contents such as unfermented products and fermented with *A. oryzae* were found to have the lowest antioxidant activity. An important detail which is worth to be underlined is the perfect

correlation between total phenolic compounds and antioxidant activity in the fermented products with *A. niger*. The highest phenolic compounds and the highest antioxidant activity were simultaneously found in 10-days fermented samples using this microorganism. The same thing was also noticed with *R. oryzae* in 15-days fermented products. Many studies confirmed these facts [27,28]. Therefore, the high in-vitro antioxidant activity detected in the fermented jackfruit rags could be explained by its high total phenolic and total flavonoid content with higher contribution coming from the total phenolic content. However, the antioxidant activity cannot be attributed only to the total phenolic and total flavonoid compounds. Indeed, the results have shown small variation between phenolic and flavonoid contents and antioxidant activities. For example, phenolic and flavonoid contents of 15-days fermented products with *A. oryzae* were higher than the unfermented products but gave lower antioxidant activity. Therefore, the antioxidant activity might also depend on other compounds already present in the jackfruit or produced by the fungi and several other factors. Among these compounds, ascorbic acid and β-carotene, which can also be found in jackfruit were proven to have high antioxidant activity [29]. The decreasing antioxidant activity found in fermented products with *A. oryzae* in contrast with its total phenolic and flavonoid contents have also been previously experienced by Karimi *et al.* [30] while studying the Solid-State Fermentation effect on Pistachio hulls' antioxidant activities. The results indicated a reduction in the antioxidant activities of pistachio hulls when undergoing Solid State Fermentation (SSF). While the antioxidant activity was 50.4 % in the control, it was recorded only 32.5 % in *A. oryzae* fermented product. They concluded that this fact could be attributed to the duration of the fermentation process. Too long time of fermentation can result in nutrient exhaustion which forces the microorganisms to use the free available phenolic and flavonoid compounds as substrate for their growth. Therefore, it is important to know the optimum fermentation time fitting with each microorganism and each raw material to get the maximum bioactive compounds and their maximum activity needed.

α-Glucosidase inhibitory assay

Figure 4 below presents the α-glucosidase inhibitory activity of extracts from fermented and unfermented jackfruit rags.
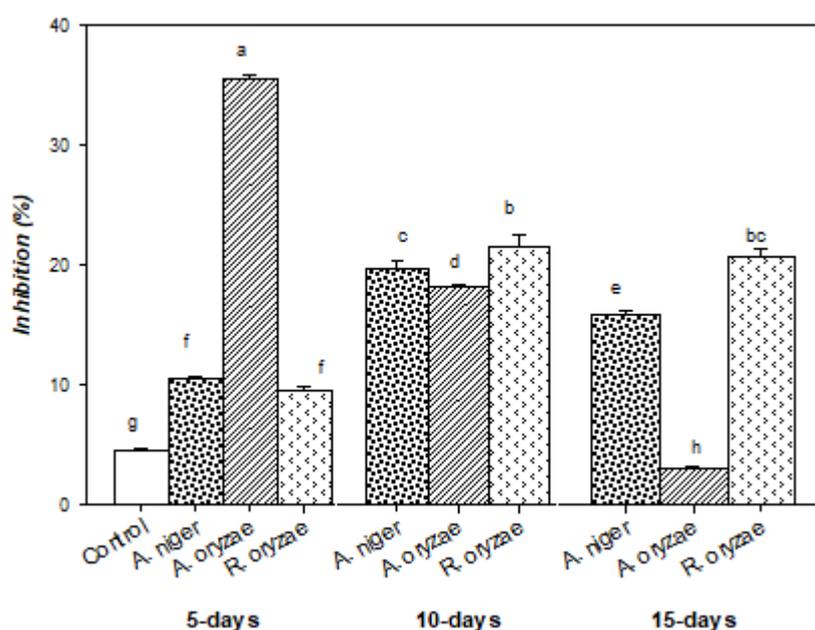

Figure 4 α-glucosidase inhibitory activity of unfermented and fermented jackfruit rags at different time with *A. niger*, *A. oryzae* and *R. oryzae*.

In general, all fermented products have higher hypoglycemic activity than non-fermented products except 15-days fermented product with *A. oryzae*. The highest α-glucosidase inhibitory activity (35.62 %) was found in 5-days fermented products with *A. oryzae*. It was significantly different from others ($p < 0.05$). It was followed by 10-days and 15-days fermented rags using *R. oryzae* with 21.61 and 20.71 %, respectively. Fermented products with *A. niger* came at the 4th ranking with 19.80 % in 10-days

fermentation. As time of fermentation increased from 5 days to 10 days, fermented products with *A. niger* and *R. oryzae* also showed an improvement in their hypoglycemic activity. However, a decrease started to be noticed at 15 days. On the other hand, *A. oryzae*, after a sharp increase of hypoglycemic activity at 5 days fermentation started decreasing from 10 days onwards with a dramatic decline at 15 days fermentation.

The highest hypoglycemic activity obtained in 5-days fermented product with *A. oryzae* can find a good explanation in previous study in which a 5-days fermentation of tryptic soy broth medium using the same fungi gave the highest α-glucosidase inhibitory activity [31]. In the same study, the results of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis indicated that the purified α-glucosidase inhibitor was a tripeptide, Pro-Phe-Pro, with the molecular weight of 360.1 Da. Except the case of *A. oryzae*, a small correlation as previously seen with antioxidant activity was found between phenolic and flavonoid content and hypoglycemic activity. An observation of **Figures 1**, **2** and **4** showed that the samples with highest phenolic and flavonoid content also showed high hypoglycemic activity. This revealed potential hypoglycemic properties from phenolic and flavonoid compounds. This was confirmed by Sarian *et al*. [32] in their study. Investigating this fact, they found that the hypoglycemic property of phenolic and flavonoid was linked to the total number and the configuration of their hydroxyl groups. The presence of C-2-C-3 double bond and C-4 ketonic groups were found to play an important role.

On the other hand, as it can be observed in **Figure 4**, the high hypoglycemic activity of fermented products with *A. oryzae* started decreasing dramatically over time to finally reach a lower level than the unfermented product (control). This implies that the α-glucosidase inhibitor produced by *A. oryzae* has either a short lifespan or can be uptake and metabolized by this microorganism when nutrient become exhausted due to longer time of fermentation. The same thing can also be applied to *A. niger* and *R. oryzae* which hypoglycemic activities started decreasing after 10 days fermentation. No previous studies have been seen specifically showing such an effect. However, the findings of Adetuyi and Ibrahim [33] about the decrease of bioactive compounds and their activities in fermented products following the time of fermentation can also be applied to our experiment. As fermentation time becomes too long, the phenolic and flavonoid compounds as well as the peptide and other bioactive compounds responsible for the hypoglycemic and other bioactivities can be used by the microorganisms in compensation of the lack of nutrients and to face stress. Other reasons could be the synthesis of enzymes capable of oxidizing α-glucosidase inhibitors or substances which interfere with the absorbance measurement in the laboratory. This phenomenon might need further studies. Understanding it could probably be of great interest for science.

Conclusions

The effect of Solid-State Fermentation (SSF) using 3 fungi on the antioxidant and hypoglycemic activities of jackfruit rags was investigated in this study. The total phenolic and total flavonoid contents which are responsible for most of these activities were first determined. They were found to be higher in fermented products than unfermented. The highest phenolic contents were found in 10-days and 15-days fermented product using *A. niger* and 15-days fermented product using *R. oryzae* with 14.16, 14.08 and 13.54 mg GAE/g D.S, respectively. They were 3 times higher than the value found in unfermented products (4.83 mg GAE/g D.S). On the other hand, flavonoid content was also revealed to be higher in fermented products than unfermented. The highest content was obtained with *A. niger* in 15-days fermentation (31.79 mg QuE/g D.S) and *R. oryzae* in 15-days fermented products (31.18 mg QuE/g D.S). They were 2 times higher than the value of unfermented product (15.08 mg QuE/g D.S). Antioxidant activities were correlated with the amount of total phenolic and total flavonoid contents. The fermented products with the highest total phenolic and flavonoid contents also gave the highest antioxidant activity. Products fermented by *A. niger* gave the highest antioxidant activity (76.43 %) after 10 days of fermentation which was perfectly correlated to the total phenolic compounds at the same time. The SSF was also able to significantly increase the hypoglycemic activity of jackfruit rags. The highest activity was found in 5-days fermented product with *A. oryzae* with a value of 35.62 % which was 8 times higher than the activity of the unfermented product (4.52 %). Therefore, this research has revealed that Solid State fermentation (SSF) could increase total phenolic and total flavonoid contents and thereby significantly improve the antioxidant and hypoglycemic activities of plant by-products. This area has great potential to expand soon due to its ability to make underutilized plant wastes incredibly useful in promoting and protecting human health against cancer and diabetes.

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