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The Effect of Angkak Addition Towards DPPH Antioxidant Activity and Yields in Mixed Angkak-Red Ginger Extracts

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Abstract

Angkak and red ginger, which represents West Borneo's Chinese ethnic group's herbal cooking, were studied to produce antioxidant activity through shogaol along with monacolin K and respond to DPPH (2, 2-diphenyl-1-picrylhydrazyl) test. This research aims to combine the two ingredients into extracts and observe how they affect the yields and IC50 value. Methods were started with the extraction of both materials in a variety of simplicia powder weights using 800 rpm stirring speed at 60°C for 120 minutes. The extracts were then tested for percentage yield (%), Thin Layer Chromatography (TLC), and half-maximal inhibitory concentration (IC50) value. IBM SPSS Statistics 25 analysis was also carried out at the end for yields and IC50 values data. Results showed that the angkak addition affected the IC50 value without giving any significant impact on each yield amount (p-value>0,05). Increased angkak in the variations led to the IC50 values decrease, ranging from 122.996 ppm (Mix 1) to 225.399 ppm (Mix 3). Angkak's pigments and red ginger's polyphenol compounds which provide antioxidant ability were found to occupy Mix extracts based on the TLC profile. In conclusion, angkak decreased the DPPH antioxidant activity of its extract combination with red ginger, but did not affect the yield percentage.

Keywords: Angkak; Antioxidant; DPPH; Red ginger; Yield.

INTRODUCTION

Ayam angkak soup, an herbal cooking from West Borneo's Chinese community which consists of angkak (*Monascus*-fermented *Oryza sativa*; widely known as "Red Yeast Rice" internationally) and ginger is renowned for its efficacy in illness recovery. As regards that, researchers have proven that shogaol and gingerol as polyphenol compounds in ginger and monacolin K in angkak produce antioxidant activity leading to the reduction of Reactive Oxygen Species (ROS) mediated by interleukin-1B (IL-1B) and lipid peroxidation in human chondrocyte cells (Promdam & Panichayupakaranant, 2022; Zhu *et al*., 2019). Those findings are worth to drug development which fits the pattern "back to nature" in society believing that natural medicines are relatively safer than synthetic drugs (Dorly, 2005). In order to maximize the antioxidant activity, ginger can be replaced with red ginger (*Zingiber officinale* var. *rubrum*), according to an in vitro study showing a more significant lipid peroxidation activity in mice's brains. Besides, red ginger's IC_{50} antioxidant levels have been found at 10.35 ppm and 44.48 ppm, as strong as angkak's, which reaches 35.37 ppm (Hasim *et al*., 2018; Illiyyin Akib *et al*., 2016; Munadi, 2020).

An appropriate extraction method for optimal antioxidant and yield. Monacolin K is thermal-sensitive and degrades at 85-121°C (Srianta *et al*., 2014). Extraction at 60°C for 120 minutes at 110 rpm stirring speed has shown an increase in the number of monacolins up to 1760 µg/g (Singgih *et al*., 2014). 6-shogaol, which was found to contribute antioxidants better than 6-gingerol is contained maximally in dry ginger extracted at 40°C, but an increase to 60°C in other studies correlated to higher antioxidant levels; therefore, the maceration method is chosen for this research (Dugasani *et al*., 2010; Ereifej *et al*., 2016; Guo *et al*., 2014). Despite the thermolabile-friendliness, efficiency should be attained as the method needs 24 hours of soaking and remaceration. Extraction temperature is thereby added to soften the plants' cell wall for easier solvent penetration and better bioactive components dissolution, followed by stirring (kinetic) to increase the mass transfer coefficient and convective mass transfer rate that facilitates the extraction process leading to yield increase. This method also outperforms other extraction types, such as reflux and soxhlet (Cha *et al*., 2020). Studies using more significant agitation averaged better dispersion, such as the curcumin nanoparticles tested at 400, 800, and 1200 rpm; and the use of 600-800 rpm in *Monascus spp.* fermentation which produced more

optimal pigments than 1200 and 300 rpm. Another corroborative research found that *Sargassum fluitans* (seaweed) extracted at 800 rpm allowed more significant secondary metabolites and solvents exchange (Akman *et al*., 2022; Gutiérrez *et al*., 2017; Manan, 2017). Thus, extraction at 60°C, 800 rpm for 120 minutes, using 96% ethanol, is chosen, since prior research found that it correlated with the increase in antioxidants and obtained up to 13.48645% yields (Putri *et al*., 2021).

Combining plant extracts with strong IC_{50} values does not always enhance antioxidant activity. For instance, binahong and basil extracts improved from 51.57 ppm and 42.34 ppm to 24.33 ppm, while cherry leaves and bark improved from 83.149 ppm and 19.632 ppm to 11.148 ppm, while the Dutch teak and kaffir lime leaf extracts combination which each had an IC_{50} value of 35.92 ppm and 48.70 ppm at a volume of 120 µg decreased after being combined in any variation (Himawan *et al*., 2021; Nadhira *et al*., 2018; Taswin & Nurjana, 2021). On the other hand, research on the yield of combined extracts has never been carried out; however, it was found that yields negatively correlate with antioxidants (Braga *et al*., 2016). It occurs because antioxidant levels also depend on the synergy of interactions between the combined plant ingredients. The interaction of statins (monacolin K) as the main ingredient in angkak and ginger's shogaol in previous in vivo studies were synergistic and no interactions that diminished the efficacy of the treatment were found based on Stockley's Herbal Medicine Interaction (Heeba & Abd-Elghany, 2010; Williamson *et al*., 2009). As a confirmation, this research aims to check whether the yields and IC_{50} values of angkak and red ginger extracts combination can be maximized by the both interactions, using the DPPH method as it suits plants with phenolic compounds and was ever found to respond to angkak as well. In regard to Chinese herbalists' medication dose, the amount of dry ginger powder used in the extraction ranges from 10-40g and angkak ranges from 14-55g (Zhang *et al*., 2022; Zubaidah *et al*., 2015).

MATERIALS AND METHODS

Materials

The plant materials were red ginger simplicia powder (*Zingiber officinale* var. *rubrum*), which was taken from Agradaya® brand whose production took place in Yogyakarta city, Indonesia with P-IRT number "5103404040184-27" and production code "5CM02220886", while angkak was taken from SU® Brand imported by PT Global Buana Mandiri (BPOM RI ML 219309046124); the other supportive materials and instruments were 96% ethanol pro-analysis (Merck™ 1.00983.2500), DPPH (2,2-diphenyl-1-picrylhydrazyl) powder (Tokyo Chemical Industry CO.LTD D4313), hotplate stirrer (DLAB™ MS-H280-Pro), buchner funnel (ROCKER™ Chemker 300), ascorbic acid (CSPC WeiSheng Pharmaceutical (Shi Jia Zhi Jiang) Co. Ltd), acetone pro-analysis (Smart-Lab® 180121001), toluene pro-analysis (Smart-Lab® 210121001), glacial acetic acid (J.T.Baker® 0000204534), silica 60 F254 plate (Supelco® 1.05554.0001), methanol pro-analysis (Merck™ 1.06009.2500), ethyl acetate pro-analysis (Merck™ 1.09623.2500), rotary evaporator (Heidolph™ type Heizbad Hei-VAP serial no. 517-61000-00-0), oven (Papalolo™), UV light (CAMAG®), UV-Vis spectrophotometer (Shimadzu® UV-2450 serial no. A10834802090), and IBM SPSS Statistics 25 software.

Procedures

Red Ginger and Angkak Extraction

Angkak was milled and sifted as well as red ginger powder in sieve no.40. Both materials were then weighted and mixed in different variations (Table 1), then extracted using 96% ethanol (1:10) by hotplate stirrer set to 800 rpm rotating speed at 60°C for 120 minutes (Kementerian Kesehatan Republik Indonesia, 2017; Putri *et al*., 2021).

Table 1. Angkak and red ginger extraction composition.

Extract Variation	Angkak Weight (g)	Red Ginger Weight (g)	Total Weight (g)	96% Ethanol (mL)
Angkak	30	0	30	300
Red Ginger	0	30	30	300
Mix 1	14	30	44	440
Mix 2	30	30	60	600
Mix 3	55	30	85	850

Extraction was carried out in 3 replications. Each macerate obtained was filtered using a Buchner funnel, then concentrated using a rotary evaporator and heated in an oven at 50°C to obtain thick extracts (Akman et al., 2022; Gutiérrez et al., 2017; Kementerian Kesehatan Republik Indonesia, 2017; Manan, 2017)

Yields Calculation
Each percentage yield (%) of the extracts was calculated using mathematical formulation as follows (Kementerian Kesehatan Republik Indonesia, 2017).

$$\text{Percentage Yield (\%)} = \frac{\text{Extract Weight (g)}}{\text{Simplicia Powder Weight (g)}} \text{x } 100\%$$

Thin Layer Chromatography (TLC) Profile
Each extract was diluted with a small amount of 96% ethanol to be spotted on a 6.5 x 1.5 cm silica plate 60 F254 using a capillary tube. The mobile phase used for Angkak was ethyl acetate: methanol: aqua (7:1:1) added with one drop of glacial acetic acid to detect pigments, toluene: acetone (9:1) was used for Red Ginger to detect its phenolic compounds, whereas Mix extracts were observed using both mobile phases. The plate containing compounds separated by toluene: acetone (9:1) was sprayed with the vanillin-sulfuric acid reagent. Spots of all plates were then examined under UV light at 254 nm and 366 nm wavelengths (Kementerian Kesehatan Republik Indonesia, 2017). Later, the whole elution was repeated to be stained using 0.2% DPPH solution.

Antioxidant Activity Measurement
Antioxidant activity was measured using the DPPH method standardized using ascorbic acid. 500 ppm of each extract stock solution was concentrated into 30, 60, 90, 120, and 150 ppm. 3 mL of each was later pipetted to 6 mL of 45 ppm DPPH solution. The incubation took 30 minutes at room temperature. The absorbances at the maximum wavelength were then measured using a UV-Vis spectrophotometer.

Data analysis
All data obtained were analyzed descriptively. Percentage yields (%) and IC_{50} values were analyzed statistically using IBM SPSS Statistics 25 afterward.

RESULTS AND DISCUSSION

Results and Discussion should be written as a series of connecting sentences, however, a manuscript with a long discussion should be divided into subtitles. Results should be clear and concise.

The Organoleptic Characteristics of The Obtained Extracts
Organoleptically, Angkak and Mix 3 were characterized by thicker textures, Mix 1 and Red Ginger tended to be more fluid, while Mix 2 was in between. Red Ginger extract was yellowish brown or dark brown color with a spicy aroma, whereas Angkak and entire Mix extracts were dark red and showed no significant color differences with spicy and bitter scents.

Extracts' Yields Calculation and The Data in Graph Form
Each replication was categorized at a good precision level, based on the acceptance range limit, following the CV Horwitz conversion for yield testing methods for this case, which could be extended to 8%. Roughly seen, Mix 2 yielded the highest (Figure 1). However, the yields of all extracts did not differ significantly and categorized as good since they were over 10% (Table 2).

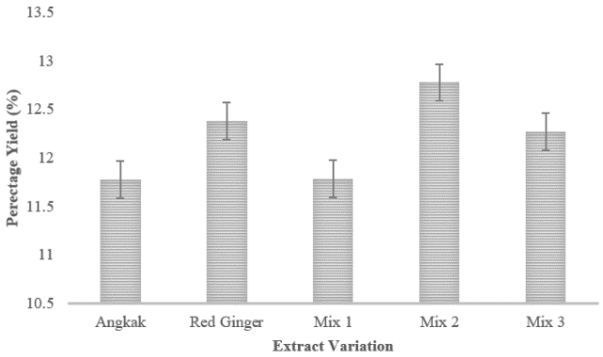

Figure 1. Extracts' yield graph.

Table 2. Obtained extracts' yields

Extract Variation	Average Yield (%)	RSD (%)
Angkak	11.775	6.282
Red Ginger	12.381	3.183
Mix 1	11.783	3.695
Mix 2	12.778	3.023
Mix 3	12.268	2.214

SPSS Analysis Result for The Yields' Data
SPSS analysis of the yield data met the normality and homogeneity requirements (p-value> 0.05). Further results from ANOVA showed an insignificant relationship between extract variation and the percent yield gain (p-value> 0.05).

Thin Layer Chromatography Test Results of Extracts Containing Red Ginger
The stains were mostly purple and brownish in color after being sprayed using vanillin-sulfuric acid, which could be estimated as terpenoid and polyphenol groups. All stains on the whole extracts also reacted to 0.2% DPPH spray, giving bright yellow color in purple background, indicating that the extracts were able to provide antioxidant activity (Figure 2).

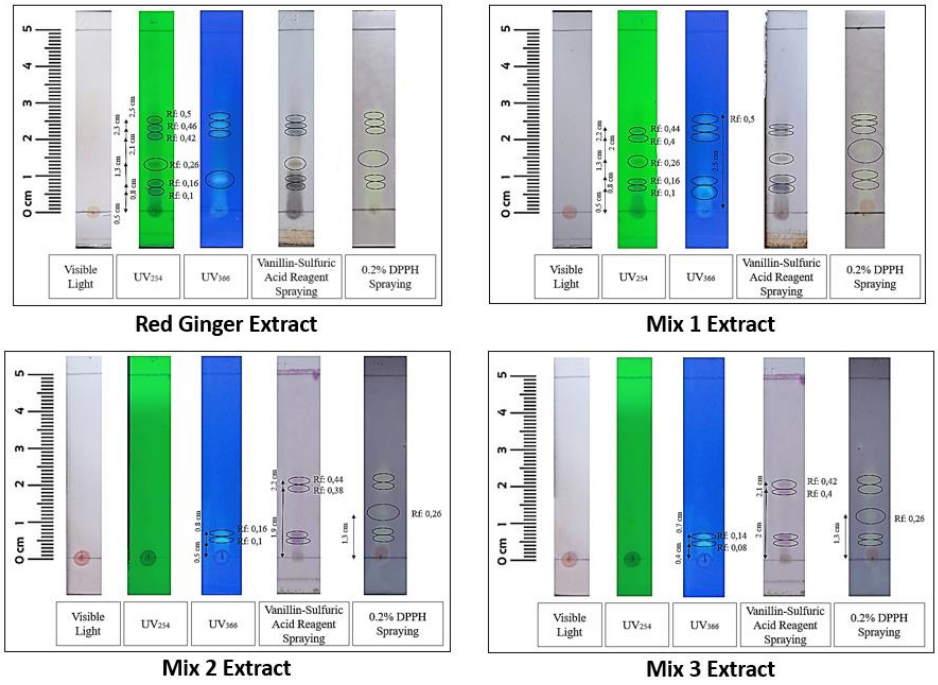

Figure 2. TLC results for chromatogram profile of red ginger's specific substances in Red Ginger extract, Mix 1, Mix 2, and Mix 3 extract using toluene: acetone (9:1) mobile phase.

Thin Layer Chromatography Test Results of Extracts Containing Angkak

All the extracts containing angkak gave 3 stains when being eluted, consisting of red and yellow pigment. Each of the Mix extract reacted to give bright yellow color change with purple background after being sprayed with 0.2% DPPH as it contained polyphenol compounds from red ginger, except the Angkak single extract (Figure 3).

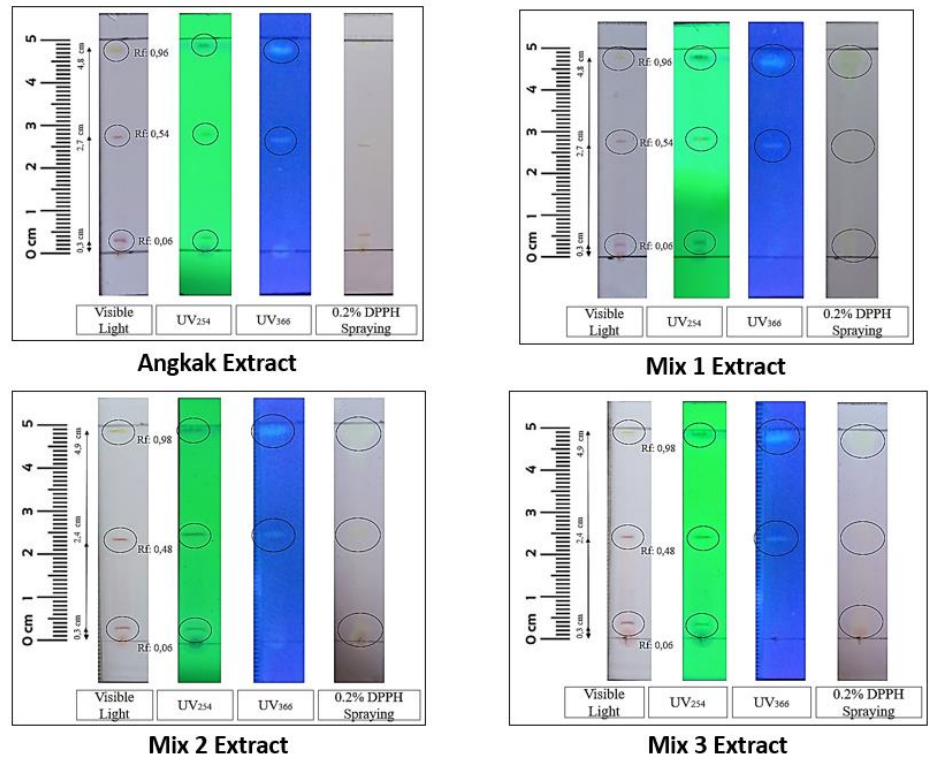

Figure 3. TLC results for angkak pigment detection in Angkak extract, Mix 1, Mix 2, and Mix 3 extract using ethyl acetate: methanol: aqua (7:1:1) added with 1 drop of glacial acetic acid mobile phase.

Antioxidant Activity (IC_{50}) of Each Extract and The Data in Graph Form

Data obtained from the antioxidant activity measurement on a variety of extracts showed that increasing the amount of angkak made the antioxidant activity decrease (Figure 4). Angkak was tested in two different concentrations, since it was first suspected that the initial concentrations used, which were 30-150 ppm were not optimal. Subsequently, an increase in the concentrations to 300-500 ppm was carried out to revalidate the method. However, it turned out to have an insignificant impact on the IC_{50} values as well. They were getting smaller and became negative in inhibition instead (Table 3). Later, it was concluded that the angkak used might not be suitable to be detected on DPPH method since it usually comes from different product processing in marketing. Therefore, it could result in a different contained composition which makes it not the same as the angkak used in other researchers' journal references whose antioxidants were detectable in the DPPH method. The precision of test replication was categorized as good, based on the established criteria for assays in the Codex Alimentarius involving the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) as well as analytical method validation guidelines from the United Nations Office on Drugs and Crime (UNODC), which tolerates up to an RSD limit of 20%.

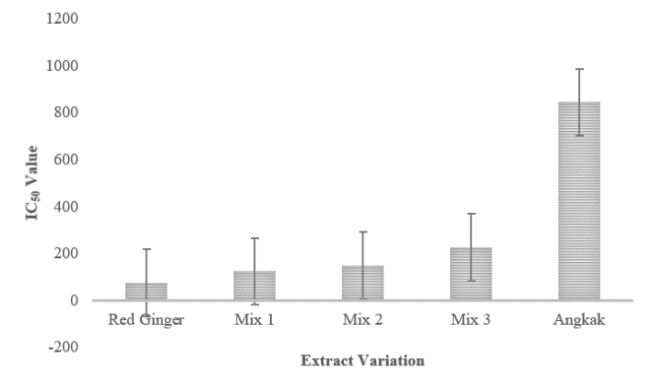

Figure 4. Extracts' IC_{50} value graph.

Table 3. Antioxidant activity measured on each extract.

Extract Variation	Average of IC50 Value (ppm)	Category (Farmawaty *et al*., 2019)	RSD (%)
Angkak (30-150 ppm)	843.942	No activity	3.353
Angkak (300-500 ppm)	-2515.247	No activity	-44.204
Red Ginger	73.973	Strong	13.870
Mix 1	122.996	Modrate	7.918
Mix 2	147.966	Moderate	12.001
Mix 3	225.399	Moderate	7.094

SPSS Analysis Result for The Antioxidant Activity Data

Analysis of the IC_{50} values using SPSS obtained parametric data that met the normality and homogeneity requirements (p-value> 0.05); therefore the test was continued with the ANOVA method. The results showed that there was a significant effect of the various extract treatments on the IC_{50} values (p-value <0.05). When continued in the Post-Hoc method, it was found that the extract variations that produced insignificantly different IC_{50} values (p-value <0.05) were only Mix 1 and Mix 2.

Discussion

Extracted Red Ginger and Angkak Organoleptic Observation

Organoleptic observation of the extracts showed that variations with higher amounts of angkak, such as Angkak and Mix 3 are characterized by thicker textures, which could be affected by the use of non-glutinous rice as angkak's raw material since its higher amylose content could make the texture more solid and dry (Dutta, 2023). Conversely, extract variations containing more red ginger tended to be more fluid, such as Mix 1 and Red Ginger. The reason could come from the presence of essential oil as a previous study found that 18.080-20.797% was extracted using ethanol solvent stirred at a maximum temperature of 50°C for 1, 3 and 5 hours (Anam, 2010). The oleoresin substances which include gingerol and shogaol as phenolic components besides giving a spicy aroma, also produced a yellowish brown or dark brown color, as seen in the Red Ginger extract (Martinus & Ramadhani, 2021). On the other hand, Angkak and the entire Mix extracts were dark red and showed no significant color differences with spicy and bitter scents.

Extracts' Yields

In the yields' calculation, Red Ginger extract was found to produce an amount close to another study's extract using conventional maceration, which was 13.48645% (Table 2) (Putri *et al*., 2021). The results even exceeded several other studies which only produced 4.10% and 8.66% yields by that conventional technique with the same ethanol percentage (Amalia *et al*., 2021; Sofyah *et al*., 2022). It proved that thermal addition could increase the extraction's mass transfer coefficient which affects diffusion and agitation increasing the solid diffusion rate

from the bulk liquid to the solid particles due to increased turbulence and decreased liquid boundary layer (Agu *et al*., 2021). Therefore, in a shorter time, the thermal kinetic maceration applied was able to provide similar yields to conventional maceration which takes up to 3 days.

The fineness of the simplicia particles also affected the yield, resulting in different values, despite the same extraction method and solvent. Extraction and yield rates increase when the particle size is smaller. The small size is associated with larger interfacial area from the solids contained within. On the other hand, the solvent requires a minimum distance to penetrate the solid particles for extraction. The larger interfacial area contributes to increased pore diffusion between the solute and the solvent, resulting in more effective penetration. Larger particles have limited contact surface area that inhibits solvent penetration and solute diffusion. Consequently, the number of extracted particles will be transported less to the surrounding solution. In other words, particles with larger diameters decrease the rates of diffusion and extraction (Agu *et al*., 2021).

Precision between replications was also examined based on the relative standard deviation (RSD) (Table 2). The acceptance range limit for this case could be extended to 8%, following the CV Horwitz conversion for yield testing methods (Riyanto, 2014). Therefore, each replication was categorized at a good precision level. The sensitivity of extract components could affect the yields of each extraction replication. For example, *M. purpureus* pigment, one of the angkak's metabolites, is sensitive to temperatures over 50°C. Hence, the extract drying and storing process requires attentiveness to ensure that none of the extracts are exposed to excessive heat (Abdollahi *et al*., 2021).

SPSS analysis of the yield data met the normality and homogeneity requirements (p-value> 0.05). Further results from ANOVA showed an insignificant relationship between extract variation and the percent yield gain (p-value> 0.05), yet some yields clearly seemed higher when processed into graph form (Figure 1). It showed that the active component's were extracted more since the solvent affinity for each substance and the components interaction in mixed extracts could affect the yield amount. Moreover, contaminants, such as bromine and iodide as volatile which are often found contaminating various products could happen and affect the yield amount as well (Mesko *et al*., 2016). Various types of substances could be extracted because ethanol is categorized as a universal solvent. Its hydroxyl group makes it appear as a polar solvent. Nonetheless, it can extract non-polar components such as alkanes and cycloalkanes because of the non-polar end on the alkyl group. A small quantity of aromatic components can also be extracted because they can form fairly weak hydrogen bonds. In addition, alkene molecules are able to be extracted slightly, as well as slightly polar aromatic components.

Thin Layer Chromatography (TLC) Test Results

During TLC, the eluent used for the chromatogram profile of red ginger's specific substances tended to be non-polar. It consisted of toluene and acetone whose polarity indexes are sequentially 2.3 and 5.4, making it more suitable for non-polar compounds, such as gingerol and shogaol (Schirmer, 1991). The separated compounds on each extract containing red ginger showed similar characteristics, the stains were mostly purple and brownish after being sprayed using vanillin-sulfuric acid, which could be estimated as terpenoid and polyphenol groups (Figure 2). Stains with less color intensity after reagent spraying indicated that the separated compound had a side chain with fewer carbon atoms, or a saturated, straight and long chain. The spots of the Red Ginger extract at Rf 0.1 and 0.26 which reacted to vanillin-sulfuric acid and were clearly visible under UV_{254}, whereas become invisible under UV_{366} as they were not categorized as fluorophore (fluorescent chemical compounds that can re-emit light upon light excitation). It fits the characteristics of shogaol and gingerol (Wang, 2020). However, comparative standards or FT-IR, LC-MS, and NMR tests in the following research were needed to accurately estimate the identity of each compound contained.

Extracts containing the same ingredients in different quantities could result in different stain characteristics, showing that composition interaction can form varieties of substances. The TLC separation of Mix 1 extract showed the same Rf value as in the Red Ginger extract at 0.5 (Figure 2). However, it was merely visible under UV_{366} and became unclear after being sprayed using vanillin-sulfuric acid, denoting the presence of polysaccharides, decalin derivatives (monascumic acid), or other components such as monascopurpurone and monacolin K which did not react to vanillin-sulfuric acid (Santebennur *et al*., 2016; Zhu *et al*., 2019). The other compounds that were clearly detected, both under UV_{254} and UV_{366}, as well as the vanillin-sulfuric acid reagent spray could be the components of red ginger or angkak which are classified as terpenoids, phenols, or organic acids (National Environmental Research Institute (NERI), 2022; Noman *et al*., 2020; Ramya *et al*., 2022). Spots with the same Rf value but different characteristics to every other extract were also detected in Mix 2 and Mix 3 extracts, respectively at 0.26 and 0.44 as shown in the (Figure 2). All stains on the whole extracts reacted to 0.2% DPPH spray, giving a bright yellow color on a purple background, indicating that the extracts were able to provide antioxidant activity.

TLC for the angkak pigment detection was then carried out using another mobile phase which tended to be polar, namely a mixture of ethyl acetate, methanol, water, and acetic acid, whose polarity indexes are

sequentially 4.3, 6.6, 9, and 6.2 (Schirmer, 1991). Any visualization reagents were not used because each type of the separated pigments will show its color and they are categorized as azaphilone polyketide which will not react to any reagents (Kementerian Kesehatan Republik Indonesia, 2017). Therefore, only 0.2% DPPH spraying was applied to determine the antioxidant activity qualitatively. All the extracts containing angkak gave 3 stains when being eluted. The red pigment of the first stain was thought to be rubropunctamine, while the second was monascurobramine, based on another previous studies which showed the polarity of the two substances based on log P. The yellow pigment could be monascin or ankaflavin which are more non-polar (Figure 3) (Shaleha *et al*., 2022). It is a fluorophore and has a chromophore group - hence the blue glow stains under UV366. The more hydrogen bonds it possessed the brighter its fluorescence was than that of red pigments (Gai *et al*., 2018). Each of the Mix extract reacted to give bright yellow color change with purple background after being sprayed with 0.2% DPPH as it contained polyphenol compounds from red ginger, except the Angkak single extract (Figure 3).

Antioxidant Activity Test Results

Later, antioxidant activity measurement using DPPH method was carried out using ascorbic acid as the positive control to ensure the procedure's accuracy. The IC_{50} value obtained for the control substance was 10.1119 ppm, classified as "very strong" in the antioxidant category, in accordance with the fact that vitamin C is an excellent antioxidant. Therefore, it could be concluded that the work on the antioxidant activity testing stages was correct and the instruments used still worked well. The level of precision between replications of this measurement was concluded based on the relative standard deviation (RSD) as shown in (Table 3). According to CV Horwitz, the requirement for a good RSD specifically for antioxidant activity measurement using UV-Vis spectrophotometry is 5.7% or less, whereas according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), the requirement for a good RSD is 2% or less (Babili *et al*., 2020; Riyanto, 2014). Nevertheless, the established criteria for assays in the Codex Alimentarius involve the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) as well as analytical method validation guidelines from the United Nations Office on Drugs and Crime (UNODC) tolerates up to an RSD limit of 20% (Codex Alimentarius Comission, 2018; United Nations Office on Drugs and Crime, 2009). On that account, it could be concluded that the RSD values in this test were still relatively good. Several factors that can influence whether RSD is good or evil include the accuracy of the method for the tested substances, the stability of the tested substances, and the skill of the tester.

Data obtained from the antioxidant activity measurement on a variety of extracts showed that increasing the amount of angkak made the antioxidant activity decrease, contrary to the initial hypothesis which states that adding angkak can increase the extracts' antioxidant levels. It was due to the undetected antioxidant activity of Angkak, as shown in (Table 3). The first suspicion of the initial concentrations used, which were 30-150 ppm was that the existing antioxidant activity was not optimal at those concentrations. Subsequently, an increase in the concentrations to 300-500 ppm was carried out to revalidate the method. However, it turned out to have an insignificant impact on the IC_{50} values as well. They were getting smaller and became negative in inhibition instead.

The inapplicability of the DPPH method to angkak's primary substance (monacolin K) was thought to be the cause of the detection failure. It was evidenced by studies showing that the result of analysis for lovastatin, which has the same chemical structure as monacolin K, was not as accurate as the lipid peroxidation inhibition assay that showed peroxidation inhibition of up to 93% at a concentration of 132 ppm, exceeding the DPPH method with a barely different concentration (100 ppm) which only produced 30% activity due to the fact that statin group compounds primarily work as lipid lowering agents, which reduce oxidative stress as well through miR-29b increase to downregulate PA200 protein (Wang *et al*., 2017). Components other than monacolins, namely polysaccharides, GABA, and ergosterol were also found to not correlate with antioxidants in the FRAP, ABTS, and DPPH methods, only pigments had an effect, but yellow and red pigments were also not detected very strongly in the DPPH test. Tests that have been carried out only show antioxidant activity of 20-25% for red and yellow pigments at a concentration of 200-400 ppm (Amany *et al*., 2020). On the other hand, researchers who got high IC50 values for angkak primarily ferment the rice which already begins with high levels of phenolics and flavonoids. For example, rice bran from YongIn, Korea which contains 1,706 µgGAE/g phenolics and 123 µgQE/g flavonoids which respectively increased to 1,793 µgGAE/g and 518 µgQE/g after fermentation. From this study, it was also found that the antioxidant activity of rice before and after fermentation differs insignificantly (Cheng *et al*., 2016).

Fermentation duration also significantly affects angkak's content. Research shows that the content of monascin reaches its peak 20 days after culture, followed by an increase in the GABA amino acids and monascumic acid, as well as rubropunctamine which increases 10 days after culture and reaches the peak around 30 days. The content of monacolins, both in the form of lactones and acids also increases after fermentation for up to 43 days (Fukami *et al*., 2021). In addition, the size of the inoculum used can also affect the amount of monacolin K. Studies that have been conducted show that monacolin K levels of 2.881 mg/g

were obtained after 10 days of fermentation through 5% inoculum (Cheng *et al*., 2016). Thus, the fermentation procedure without official regulations could be the reason for the differences in the content and response of angkak from the DPPH method in various tests. Another example of this case is the unexplained fermentation method for angkak from Bogor IR42 rice raw material only produced an antioxidant activity of 2.64% at 10,000 ppm concentration, whereas Bogor angkak extracted in other studies produced an IC_{50} value of up to 35.37 ppm (Asben *et al*., 2020; Illiyyin Akib *et al*., 2016).

The general recipe of angkak is only a requirement to use non-glutinous rice as its raw material. Non-glutinous rice has higher amylose content, resulting in a more complex texture, thus able to prevent surface area reduction that inhibits fungal growth due to sticky texture which results in fewer pigments and fermentable metabolites. The addition of a certain amount of water during fermentation can increase the amount of amylose due to faster fungal growth, resulting in the release of amylose from gelatinized starch during the sterilization process and the decomposition of α-1,6 glycosyl bonds in starch by the glucoamylase produced by Monascus. Other metabolites, such as pigments and monacolins also increase in this treatment, but the results vary depending on the rice and the type of fungi used. If it apparently happens to the angkak used in this research, then the cause of the antioxidant activity detection failure by the DPPH method could also be caused by the interaction of the amount of amylose which exceeds the phenolics, thereby covering up the reaction of phenolic antioxidants, that even the presence of betulinic acid in angkak which is able to increase red ginger's phenolic antioxidants becomes ineffectual, proved by the reduced antioxidant activity along with the addition of angkak. Amylose even has better bonds to polyphenols than amylopectin because it has more considerable molecular weight, with the type of bond that usually occurs is in the form of hydrogen bonds. Several studies have shown that the interaction of polyphenols with amylose chains can reduce the inhibition of DPPH and α-amylase, especially if the phenolics contain only 1 hydroxyl group, such as vanillic and syringic acid, so that multiple concentrations of phenolics are needed to increase the inhibition value. Amylose itself on the other hand was found to have no antioxidants through the DPPH method (Lv *et al*., 2019). In addition, complexation between phenolics and starch can affect the granules' texture, such as the occurrence of agglomeration in rice starch after gelatination or the formation of granules with many holes, thereby affecting solubility. Amylose was extracted due to the presence of thermal and the temperature of 60°C used was found sufficient to break down granules into amylose and cause gelatination, thus forming a thick, gel-like texture (Lumdubwong, 2019). However, when being unheated, amylose has poor solubility in ethanol, thus affecting its reaction with DPPH during the dissolving process as it was found that the sample and DPPH solvent greatly determine the test results (La *et al*., 2021).

Analysis of the IC_{50} values using SPSS obtained parametric data that met the normality and homogeneity requirements (p-value> 0.05); therefore the test was continued with the ANOVA method. The results showed that there was a significant effect of the various extract treatments on the IC_{50} values (p-value <0.05). When continued in the Post-Hoc method, it was found that the extract variations that produced insignificantly different IC_{50} values (p-value <0.05) were only Mix 1 and Mix 2. However, the increase and decrease level in IC_{50} values between entire extracts was quite visible when being displayed in graphical form (Figure 4).

CONCLUSIONS

The amount of angkak added to the mixed extract of angkak and red ginger affected the DPPH antioxidant activity significantly without giving any impact to the amount of each yield. Increased angkak in the variations leaded to the decrease of IC50 values, ranging from 122.996 ppm (Mix 1) to 225.399 ppm (Mix 3). Angkak's pigments and red ginger's polyphenol compounds which provide antioxidant ability were found to be contained in Mix extracts based on the Thin Layer Chromatography (TLC) profile.

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