Science and Technology Indonesia
e-ISSN:2580-4391 *p*-ISSN:2580-4405
Vol. 9, No. 1, January 2024

Research Paper

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Combination Effect of Extracts and Pure Compounds of Endophytic Fungi Isolated from Sungkai (*Peronema canescens*) Leaves on Antioxidant Activity

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Abstract

Endophytic fungi extracts have various biological and pharmacological activities as natural antioxidants which have the potential to be developed in the pharmaceutical. Drugs made from natural substances frequently work best when they are present in large quantities. This may result in unfavorable side effects from using more of one substance at a time. This limitation can be overcome by using a combination of the extracts/pure compounds that allow synergistic interactions with strong antioxidant properties at fairly low concentrations. The purpose of this research was to determine the combined effect of endophytic fungi extracts and its pure compounds on their antioxidant activity. This research was initiated by rejuvenating eight selected endophytic fungal isolates from Sungkai leaves and morphological characterization was carried out. Each fungus was cultivated in PDB medium for 4 weeks under statistical conditions. Ethyl acetate was used to extract the endophytic fungi's liquid culture, which was then evaporated. Each endophytic fungal extract (PD1-PD8) and their five pure compounds were tested for antioxidant activity by the DPPH method. Then a combination of two and three extracts was carried out with a ratio of 1:1. The results show that the interaction of the mixture of endophytic fungi extracts and their pure compounds can be classified as synergistic (combined effect of the extracts > individual effects), additive (combined effect of the extracts = individual effect), and nothing is antagonistic (combined effect of the extracts < individual effects). The best combination in this study was PD4+PD5+PD6 (1:1:1) which could be used as a formula for further research into in vivo immunostimulant tests. Another conclusion from this research is that the pure compounds contained in the extracts have lower antioxidant activity than the extracts and do not have a synergistic effect on the combination.

Keywords

Antioxidant, Combination Effect, Endophytic Fungi Extract, Pure Compound, Sungkai

Received: 22 August 2023, Accepted: 16 November 2023
https://doi.org/10.26554/sti.2024.9.1.69-76

1. INTRODUCTION

Sungkai (*Peronema canescens*) is a tropical plant that is often found in tropical rainforests, has the synonymous name *Peronema heterophyllum* Miq. belongs to the Verbenaceae family. This plant is spread in Thailand, Malaysia and Indonesia. Sungkai leaves are the plant most trusted by the public for increasing the body's immunity related to antioxidant activity (Dillasamola et al., 2021; Rahardhian et al., 2022). Research on bioactive compounds from endophytic fungi of medicinal plants is currently attracting great attention because of their enormous expansion potential in the medical, agricultural, and industrial sectors (Gakuubi et al., 2021; Nguyen and Bhattacharya, 2022; Santra and Banerjee, 2022). Literature studies show that bioactive metabolite compounds produced by endophytic fungi can help in the discovery of new drugs (El Sayed et al., 2020; Zihad et al., 2022; Oktiansyah et al., 2023a; Tiwari and Bae, 2022).

Plant extracts and essential oils are among the many natural products that are used extensively across the world because of their antioxidant activity, which protects people against a variety of ailments brought on by oxidative stress (Aggrey et al., 2019; Sonam and Guleria, 2017; Srisuksomwong et al., 2023; Zulkefli et al., 2023). Synergistic interactions between various antioxidant chemicals in mixtures of synthetic and natural antioxidants, natural products, or mixtures of various herbal extracts or plant essential oils can increase antioxidant capacity (Basavegowda and Baek, 2021; Ben Mrid et al., 2022; Sharma et al., 2020). In this study, the same principle was applied to combinations of the extracts/ pure compounds from endophytic fungi.

According to studies, mixtures of bioactive substances are less likely to cause disease resistance than single active molecules (Sitarek et al., 2020; Vaou et al., 2022). In actuality, a mixture of chemicals having additive, antagonistic, or synergistic effects produces an extract's total activity. In certain cases, an extract's activity shows more effective result than an equivalent dosage of the separated component (Ebrahimi et al., 2023; Falakdin et al., 2023; Vaou et al., 2022). As a result, while treating medicinal plants, plant extracts or a mixture of extracts from several plants are frequently used; no one bioactive molecule is used. Synergism is the coordinated or correlated activity of two or more physiological structures, agents, or processes such that their combined effect is larger than the sum of their individual effects (Choudhury, 2022; Liu et al., 2016; Vaou et al., 2022).

Drug combinations have generally been found to be a significant aspect of antibacterial and antioxidant treatment for a number of crucial factors. Drug combinations can enhance activity through synergistic or additive interactions (Dai et al., 2022; Fazly Bazzaz et al., 2021; Murugaiyan et al., 2022). Due to the existence of numerous active components that have a variety of structures and functional groups, this pharmacological combination can overcome drug resistance. In addition, the combination of drugs can reduce the dose required so that adverse / toxic side effects can be reduced. Combinations of two or more different components in a mixture can enhance or complement each other's activities and reflect a broad spectrum of activity (Choudhury, 2022; Liu et al., 2016; Pezzani et al., 2019).

Septiana et al. (2020) tested the antioxidant activity of endophytic fungal extracts from turmeric flowers. Apart from that, they also tested the antioxidant activity of the extract mixture. The results of this research show that a combination of extracts can increase antioxidant activity as evidenced by a lower IC50 value compared to single culture extracts. In addition, the extract mixture showed a synergistic effect between endophytic fungal isolates in producing antioxidant compounds.

The results of this study provide information about the effect of a combination of endophytic fungi extracts from Sungkai leaves and their pure compounds on antioxidant activity, both synergistic and additive. It is also necessary to consider whether for further development towards antioxidant preparations using single extracts, combined extracts, pure single compounds, or combinations of pure compounds.

2. EXPERIMENTAL SECTION

2.1 Research Materials

Endophytic fungi isolates (PD1-PD8) were taken from stock cultures isolated from Sungkai leaves in 2022 and stored at the Microbiology Laboratory, Sriwijaya University. Pure compounds (C1-C5) were obtained from the isolation of endophytic fungi PD4, PD5, PD6, PD7 in 2022 and stored at the Organic Chemistry Laboratory, Sriwijaya University. The materials to conduct this study were Potato Dextrose Agar, Potato Dextrose Broth, alcohol 70%, physiological NaCl solution, ethyl acetate, methanol p.a., DPPH (2,2-diphenyl-1-picrylhydrazyl).

2.2 Research Instruments

The instruments used to avoid contamination include autoclave (Hirayama HVE350) and laminar air flow (LVG-4AG-F8). Analyze microscopic characteristics using microscope digital (Hirox RH-2000). Homogenize the media using magnetic stirrer and hotplate (Cimarec + digital SP88857105). Concentrated extract using rotary evaporator (Buchi R300+ V-300 with interface I-300 Pro + F305). Antioxidant activity test used spectrophotometer UV-Vis (Shimadzu UV-1900).

2.3 Research Procedure

2.3.1 Endophytic Fungi Rejuvenation and Morphological Identification

Rejuvenation of endophytic fungal isolates was carried out by regrowing purified endophytic fungal isolates into PDA media and incubated for 5-7 days at room temperature. Furthermore, the rejuvenation results were identified morphologically including macroscopic and microscopic characters. The following macroscopic characteristics observed were the color of the colony's surface and back, its texture (cotton, grain, powder, slimy), the existence of exudate droplets, the presence of radial lines, and the presence of concentric circles. Observing hyphae, spores, color, and other distinct traits under a microscope with a 1000X magnification is used to analyze microscopic characteristics using the slide culture method. The characters that emerge were compared to the solitary characters tested in 2022 (Pitt and Hocking, 2009; Walsh et al., 2018; Watanabe, 1993).

2.3.2 Cultivation and Extraction

The endophytic fungal culture on PDA media was cut into small pieces (6 mm in diameter) and then the 3 blocks were transferred to a 1000 mL Erlenmeyer flask containing 300 mL PDB medium. PDB media used for cultivation was 900 mL which was placed in 3 Erlenmeyer. After incubation at room temperature for 30 days, the fermented broth of each fungus was vacuum filtered to separate the mycelia. The PDB culture broth was then partitioned in a separatory funnel with ethyl acetate solvent. The ethyl acetate extract was concentrated using a vacuum evaporator at 40°C. The concentrated crude extract was then weighed and stored at 4°C for use in studying its antioxidant activity (Gupta et al., 2023; Oktiansyah et al., 2023; Widjajanti et al., 2022).

2.3.3 Antioxidant Activity Test

The DPPH radical scavenging assay was used to evaluate the extracts and pure compounds capacity to scavenge free radicals following the procedure of Sari et al. (2023). In a concentration series of 250, 125, 62.5, 31.25, 6.25 g/mL, and 0.05 mM DPPH in methanol was produced and 3.8 mL of this solution was combined with 0.2 mL of the test sample in methanol.

After complete vortexing, the reaction mixture was kept in the dark for 30 minutes. At 517 nm, the mixture's absorbance was determined spectrophotometrically. Ascorbic acid served as the benchmark. The percentage of DPPH radical scavenging activity was calculated by the formula:

$$\% \text{ Inhibition} = \frac{A_C\text{-}A_S}{A_S} \times 100$$

Ac = Absorbance of control

As = Absorbance of samples

2.3.4 Effect of the Extract/Pure Compound Combination on Antioxidant Activity

Each of the endophytic fungi extracts (PD1-PD8) and pure compounds (C1-C5) were prepared at a concentration of 250 μg/mL as much as 5 mL. Combination effect tests were carried out by combining two or three extract components in a 1:1 ratio. Nine extract combinations were prepared through tests 1-9. Each test measured the antioxidant activity of the single components and their combinations at a concentration of 250 μg/mL. Each was calculated % inhibition and adjusted for the occurrence of color changes. The more yellow the test solution indicates the more active the antioxidant. Combination effects are grouped into three according Pezzani et al. (2019).

Additive : OE (Da, Db) = OE (Da) + OE (Db)

Synergis : OE (Da, Db) > OE (Da) + OE (Db)

Antagonis : OE (Da, Db) < OE (Da) + OE (Db)

OE = observed effects

Da, Db = doses of the X-sample and Y-sample

3. RESULTS AND DISCUSSION

Eight endophytic fungi selected in previous studies were taken from stock cultures and rejuvenated to obtain fresh cultures. Morphological identification has been carried out and endophytic fungal cultures have the same identity as previous studies. The eight endophytic fungi isolates were *Pythium elongatum* (PD1), *Paecilomyces variabilis* (PD2), *Pythium afertile* (PD3), *Aspergillus niger* (PD4), *Trichoderma asperellum* (PD5), *Lasiodiplodia theobromae* (PD6), *Penicillium oxalicum* (PD7), *Trichoderma sp.* (PD8). After the rejuvenation stage of the eight endophytic fungi selected from our previous study (PD1-PD8), they were cultivated in PDB growth media for an incubation period of 4 weeks so that the endophytic fungi could produce their secondary metabolites optimally. Liquid-liquid extraction of endophytic fungal culture media in ethyl acetate solvent followed by evaporation produces a concentrated extract of ethyl acetate (PD1-PD8).

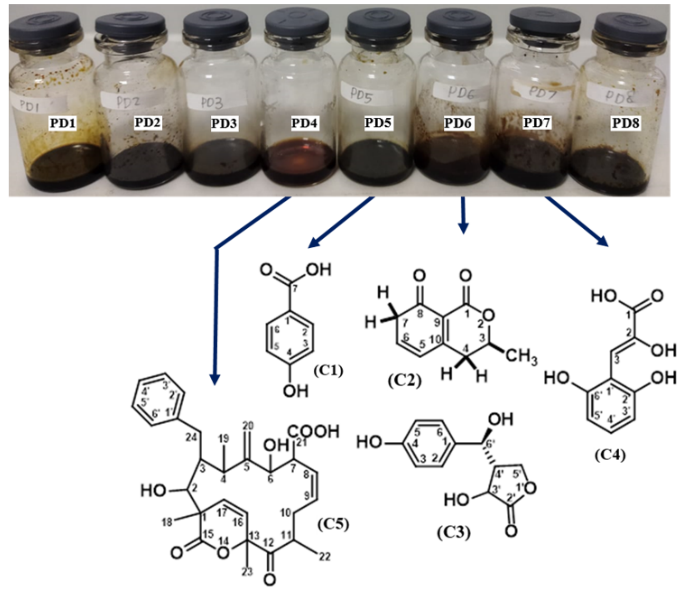


Figure 1. Ethyl Acetate Extract of Endohytic Fungi (PD1-PD8) from Sungkai Leaves and its Pure Compounds (C1-C5)

Our previous research reported five pure compounds namely C1-C5 which were isolated from four endophytic fungi namely PD4, PD5, PD6, and PD7 respectively. Ethyl acetate extract of endohytic fungi (PD1-PD8) and its pure compounds (C1-C5) are shown in Figure 1.

3.1 Antioxidant Activity of Ethyl Acetate Extracts and its Pure Compounds

The antioxidant activity test using the 1,1-diphenyl-2-picrylhy drazyl (DPPH) method is based on the ability to scavenge DPPH radicals. The antioxidant activity of a compound is indicated by the inhibition of DPPH absorption by calculating the percentage of inhibition of DPPH absorbance at a wavelength of 517 nm. The antioxidant activity of ethyl acetate extract and its pure compounds from the endophytic fungus of Sungkai leaves is shown in Table 1.

Table 1 shows that the ethyl acetate extract of the endophytic fungi PD5, PD6, PD7 has high antioxidant activity equivalent to its host plant. Five pure compounds (C1-C5) isolated from four endophytic fungi (PD4-PD7) showed lower antioxidant activity than the original ethyl acetate extract of endophytic fungi and methanol extract of Sungkai leaves. This may be due to the presence of antioxidant compounds in the extract which have not been isolated in this study. Another theory is that a combination of compounds (phytochemicals) would have higher bioactivity than a single component since the oxidation process uses a complicated set of processes. The combination of bioactive chemicals in an extract has the power to affect a variety of targets, and an antioxidant molecule's effectiveness is dependent on other compounds (Sonam and Guleria, 2017).

Table 1. Antioxidant Activity of Ethyl Acetate Extract and its Pure Compounds from the Endophytic Fungus of Sungkai Leaves (Ascorbic Acid and Host Plant as a Comparison)

Isolate Code	IC50 Extract (μg/mL)	Pure Compounds Code	IC50 Pure Compounds (μg/mL)
PD 1	246.3		
PD 2	> 250		
PD 3	58.4		
PD 4	81.6	C5	> 250
PD 5	22.2	C1	84.3
PD 6	23.3	C2 & C3	> 250 & 26.8
PD 7	27.1	C4	32.5
PD 8	51.6		
Ascorbic acid	16.7		
Host plant (Methanol extract of Sungkai leaves)	19.4*		

*Elfita et al. (2022)

3.2 The Combination Effect of Endophytic Fungi Extracts on Antioxidant Activity

In a previous study, we isolated 16 endophytic fungi from Sungkai leaves (PD1-PD16). In this study, we selected eight endophytic fungi (PD1-PD8) based on their antioxidant activity which were categorized as very strong, strong, and moderate and has good growth in PDB medium. Eight other endophytic fungi (PD9-PD16) had varying antioxidant activities, namely IC50 > 250 μg/mL (inactive: PD10, PD11, and PD15), IC50 > 100 μg/mL (weak: PD9 and PD16), IC50 = 50 – 100 μg/mL (moderate: PD12 and PD14), and IC50 = 36.7 μg/mL (strong: PD13). Each selected endophytic fungi extract was combined two or three components with 1:1 composition and was tested for antioxidant activity. The effect of extract/ pure compound combination on antioxidant activity was measured in % inhibition. This was done to facilitate the observation of the combination effect as the first step of this research. The results of this combination test for antioxidant activity are shown in Figure 2.

DPPH has a strong absorption at a wavelength of 517 nm with a dark violet color. The presence of lone electrons causes the DPPH radical (in purple) to be highly reactive to capture electrons or other hydrogen radicals to become stable diamagnetic molecules. A reduction in the absorption value of DPPH occurs when lone electrons pair up due to the presence of an antioxidant (RH) to form a yellow stable DPPH.

The ability to scavenge free radicals from pure extracts/ compounds is determined by their ability to donate hydrogen atoms in the sample as indicated by the decolorization of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution. Determination of DPPH radical scavenging activity was carried out by measuring the absorbance of the remaining DPPH radicals with a UV-vis spectrophotometer at λmax 517 nm. DPPH is purple in methanol solution and fades to yellow in the presence of antioxidants. The yellower the methanol DPPH solution, the stronger the antioxidant activity.

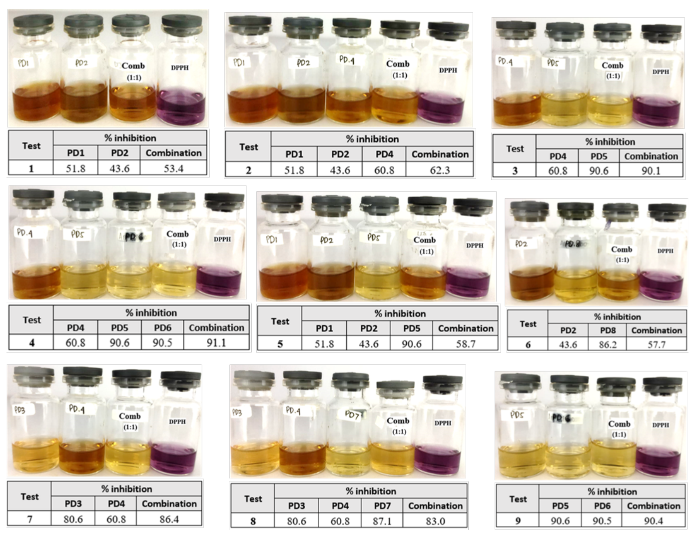


Figure 2. Effect of Endophytic Fungi Extract Combination on Antioxidant Activity (% Inhibition at a Concentration of 250 μg/mL)

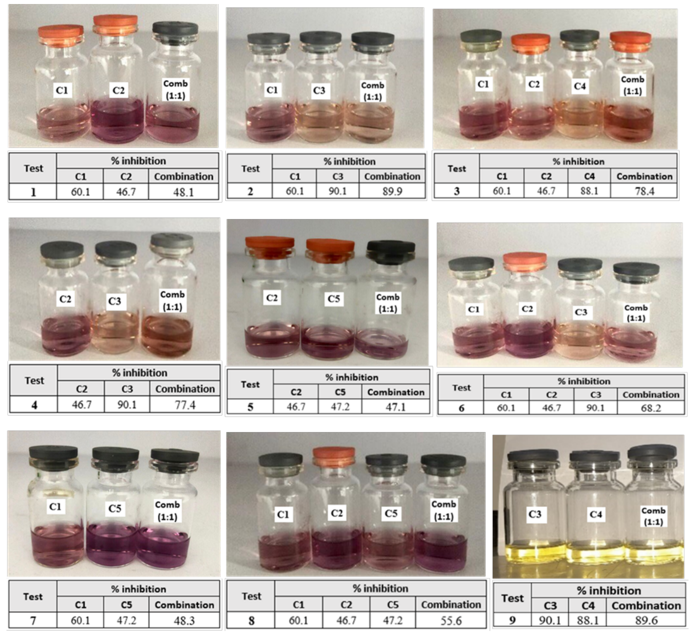
Figure 2 illustrates the combined effects of endophytic fungal extracts on antioxidant activity. These effects can be categorized as additive, antagonistic, or synergistic. If the impact of the combined components is higher than the effect of the individual components, this is known as synergism. It was identified that the combinations in tests 1, 2, 4, and 7 experienced a synergistic effect. The fact that the two chemicals' combined impact is a pure addition effect is demonstrated by their additive and non-interactive combination. It was identified that the combinations in tests 5, 6, 8, and 9 experienced an additive effect. The additive impact is determined from the separate effects using intricate mathematical algorithm formulae, rather than simply adding the effects of components A + B. Since antagonism is a lesser result than anticipated, it is considerably simpler to describe. The combined effects listed in Figure 2 do not show antagonistic effects. The combination effect in test 3 provides an equivalent % inhibition of one of the active components. This means that the PD5 extract with half the concentration in a mixed combination with the PD4 extract, still provides strong antioxidant activity. The combination of PD4 and PD5 extracts is preferred as a drug formula compared to a single PD5 extract even though it provides the same antioxidant activity. The best antioxidant activity of the extract combination was in tests 3, 4, and 9, each of which gave a % inhibition value of > 90%. Because the various components in the mixture will strengthen or complement each other's activities, the combination of extracts might lower the necessary dose and minimize undesirable or harmful side effects that may affect multiple targets.

3.3 The Combination Effect of its Pure Compounds on Antioxidant Activity

In a previous study, we have reported the isolation of five pure compounds (C1-C5) from the endophytic fungi of Sungkai leaves. The five compounds are: 4-hydroxybenzoic Acid (C1) isolated from *Trichoderma asperellum* (Oktiansyah et al., 2023a); 3- methyl-3,4-dihydro-1H-isochromene-1,8(7H)-dione (C2) and 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-γ-butyrolactone (C3) isolated from *Lasiodiplodia theobromae* (Elfita et al., 2023); 3-(2,6- dihydroxyphenyl)-2-hydroxyacrylic acid (C4) isolated from *Penicillium oxalicum* (Elfita et al., 2022); and 3-benzyl-2,6-dihydroxy-1,4,11,13-tetramethyl-5-methylene-12, 15-dioxo-14- oxabicycloheptadeca-8,16-diene-7-carboxylic acid (C5) isolated from *Aspergillus niger* (Oktiansyah et al., 2023b). Each pure compound isolated from the endophytic fungus of Sungkai leaves was combined with two or three components with a 1:1 composition and was tested for its antioxidant activity. The results of the pure compound combination test for antioxidant activity are shown in Figure 3.

The effect of the combination of pure compounds (C1-C5) from the endophytic fungus of Sungkai leaves on the antioxidant activity shown in Figure 3 can be identified that tests 1-test 9 all have additive effects. The best additive effect that results in high antioxidant activity is the combination in tests 2 and test 9 with % inhibition of 89.9 and 89.6%. Based on the results of this study, only the combination of extracts (PD1-PD8) produced a synergistic effect. This could be as a result of each extract containing several components. In varied amounts, endophytic fungus extracts may contain dozens or more distinct bioactive chemicals. When one extract controls another's absorption, distribution, metabolism, and excretion to increase their therapeutic efficacy, known as synergy effects, occur, or when all the extracts involved are individually inert but become active when combined, is known as additive action (Atanasov et al., 2021; Chan et al., 2023; Vaou et al., 2022; Zhou et al., 2016).

The same case occurs in medicinal plants. Synergistic interactions between medicinal plant mixtures or extracts are an important part of therapeutic efficacy (Ialongo et al., 2023; Komariah et al., 2023; Nutmakul and Chewchinda, 2023; Ranjutha et al., 2023). Therefore, it is necessary to assess the synergy of medicinal plant extracts or endophytic fungal extracts using exacting analytical techniques and to confirm the results in clinical studies. Additionally, the precise bioactive substances in charge of these outcomes as well as the fundamental principles governing how they interact are still poorly known (Vaou et al., 2022).



Test	% inhibition		
	C1	C2	Combination
1	60.1	46.7	48.1

Test	% inhibition		
	C1	C3	Combination
2	60.1	90.1	89.9

Test	% inhibition			
	C1	C2	C4	Combination
3	60.1	46.7	88.1	78.4

Test	% inhibition		
	C2	C3	Combination
4	46.7	90.1	77.4

Test	% inhibition		
	C2	C5	Combination
5	46.7	47.2	47.1

Test	% inhibition			
	C1	C2	C3	Combination
6	60.1	46.7	90.1	68.2

Test	% inhibition		
	C1	C5	Combination
7	60.1	47.2	48.3

Test	% inhibition			
	C1	C2	C5	Combination
8	60.1	46.7	47.2	55.6

Test	% inhibition		
	C3	C4	Combination
9	90.1	88.1	89.6

Figure 3. The Effect of the Combination of Pure Compounds (C1-C5) from the Endophytic Fungi of Sungkai Leaves on Antioxidant Activity (% Inhibition at a Concentration of 250 μg/mL)

The synergistic effect of extract combinations in test combinations 1, 2, 4, and 7 (Figure 2) can reduce the need for different extract doses in drug combinations thereby reducing side effects caused by high concentrations of one drug. However, it is not yet known about the interaction mechanism responsible for the synergistic antioxidant activity and further research is needed. Synergistic interactions are highly sensitive and dependent on the many types of chemicals combined with them as well as the relative amounts of those compounds. The antioxidant activity of single extracts, combinations of extracts, single pure compounds, and combinations of pure compounds as measured in % inhibition are listed in Table 2.

Sonam and Guleria (2017) reported on the synergistic effect of a mixture of antioxidant components from various extracts of natural ingredients. Combinations of several herbs show synergistic antioxidant activity. The synergistic antioxidant activity of green tea with several herbs supports the hypothesis that a combination formulation of plant polyherbs that have antioxidant activity (*Vitis vinifera*, *Phyllanthus emblica L.*, *Punica granatum*, *Cinnamomum cassia*, *Ginkgo biloba L.*, and

Table 2. The Antioxidant Activity of Single Extracts, Combinations of Extracts, Single Pure Compounds, and Combinations of Pure Compounds as Measured in % Inhibition

Samples group	Samples Code	% Inhibition (at 250 μg/mL)	Samples group	Samples Code	% Inhibition (at 250 μg/mL)
Ethyl acetate extract	PD 1	51.8 ± 1.07	Pure compound	C1	60.1 ± 0.43
	PD 2	43.6 ± 0.33		C2	46.7 ± 3.27
	PD 3	80.6 ± 1.12		C3	90.1 ± 0.67
	PD 4	60.8 ± 0.47		C4	88.1 ± 0.41
	PD 5	90.6 ± 0.26		C5	47.2 ± 1.26
	PD 6	90.5 ± 0.79		Test 1 (C1+C2)	48.1 ± 2.13**
	PD 7	87.1 ± 2.03		Test 2 (C1+C3)	89.9 ± 1.42**
	PD 8	86.2 ± 0.56		Test 3 (C1+ C2+C4)	78.4 ± 1.17**
Combination (1 : 1)	Test 1 (PD1+PD2)	53.4 ± 0.82 *	Combination (1 : 1)	Test 4 (C2+C3)	77.4 ± 0.53**
	Test 2 (PD1+PD2+PD4)	62.3 ± 0.19 *		Test 5 (C2+C5)	47.1 ± 1.12**
	Test 3 (PD4+PD5)	90.1 ± 0.26**		Test 6 (C1+ C2+C3)	68.2 ± 0.57**
	Test 4 (PD4+PD5+PD6)	91.1 ± 0.34 *		Test 7 (C1+C5)	48.3 ± 1.18**
	Test 5 (PD1+PD2+PD5)	58.7 ± 1.08**		Test 8 (C1+ C2+C5)	55.6 ± 2.38**
	Test 6 (PD2+PD8)	57.7 ± 2.44**		Test 9 (C3+C4)	89.6 ± 0.33**
	Test 7 (PD3+PD4)	86.4 ± 0.97 *			
	Test 8 (PD3+PD4+PD7)	83.0 ± 0.45**			
	Test 9 (PD5+PD6)	90.4 ± 0.39**			
Antioxidant standard	Ascorbic acid	93.4 ± 1.26	Antioxidant standard	Ascorbic acid	93.4 ± 1.26

*synergistic effect **additive effect

Camellia sinesis Linn.) shows a synergistic effect with green tea. It was found that the antioxidant activity increased synergistically with the action of all these ingredients in combination with green tea. Thus, a lower dose of any herb with green tea can be used. Studies report that selected individual plants contain abundant amounts of phenolics and flavanoids and their polyherbal combination with green tea was found to produce the best antioxidant activity among all the individual extracts. This can help avoid unwanted side effects due to higher doses of single herbs.

The same thing can also be seen from the combined effect of the endophytic fungi extracts of Sungkai leaves. The best synergistic effect was seen in the PD4+PD5+PD6 combination (test 4). The combined effect results in a mixture with the highest activity among all the individual extracts. Thus, this combination can help avoid unwanted side effects due to higher doses of single ingredients. The quite good effect of the additive combination of Sungkai leaves endophytic fungi extract was also seen in the PD4+PD5 combination (test 3) by giving a high % inhibition value equivalent to that of the single extract, namely PD5. Likewise in the combination of pure compounds, namely the combination C1+C3 (test 2) and the combination C3+C4 (test 9) the combination effect was obtained with a high % inhibition value equivalent to the % inhibition of single pure compounds, namely C3 and C4.

4. CONCLUSION

The results obtained in this study support the fact that the antioxidant activity of the combination extract cannot be predicted from the activity of the single extract. The pure compounds contained in the extracts have lower antioxidant activity than the extracts and do not have a synergistic effect in the combination. The best combination in this study that can be used as a formula for in vivo research is the PD4+PD5+PD6 combination (test 4). The synergistic effect results in the mixture with the highest activity among all the individual extracts. Thus, this combination formula can help avoid unwanted side effects due to higher doses of single ingredients. The additive effect that occurs in the C1+C3 combination (test 2) and the C3+C4 combination (test 9) seems to be used as a further formula compared to the single pure compound with equivalent antioxidant activity.

5. ACKNOWLEDGMENT

The authors thank to the Kementerian Pendidikan, Kebudayaan, Riset, dan Teknologi which funded this research throughout Penelitian Dasar Unggulan Perguruan Tinggi, contract number 059/E5/PG.02.00.PL/2023, derivative contract number 0193.02/UN9.3.1/PL/2023.

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