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Profiling Secondary Metabolite of Fresh and Fermented Marigold Flowers and Butterfly Pea Flowers

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ABSTRACT

Communities in Bali use "Usada" as an essential plant for traditional medicine. Utilization of a plant as herbal medicine is based on the metabolites content, such as in the marigold flower (Tagetes erecta L.*) and butterfly pea flower (Clitoria ternatea* L.*). Identification of secondary metabolites in fermented samples. This study aims to identify the secondary metabolite profiles of control extracts compared to fermented extracts. Aerobic fermentation is carried out by stacking fresh samples fot two hour in temparuture room. Extraction by percolation using n-hexane for marigold flowers and methanol for butterfly pea flowers. The extract profile was analyzed by densitometric thin-layer chromatography (TLC). Separation pattern, retention time, and m/z fragmentation with LC-MS/MS. Antioxidant activity tests and calculations of total phenol, flavonoid, and carotenoid levels were also performed on each sample. The TLC profiles of the control extract and the fermented pea flower extract were similar, but the fermented pea flower extract had thicker spots, and a new compound was formed from the TLC pattern. The control and fermented extracts had a total phenolic content ranging from 0,816 – 1,154 g GAE/100 g extract and total flavonoid content ranging from 0,067 – 0,610 g QE/100 g extract. Measurement of total carotenoid levels in fermented marigold flower extract yielded Simplicia 215,000 mg/g higher than the control extract, which was 200,265 mg/g simplicia. All control and fermented extracts had antioxidant activity ranging from 46,71 ppm (very strong) – 75,30 ppm (strong). LC-MS/MS analysis showed five compounds were identified from fermented butterfly pea flower extract, while the control identified four compounds.The fermentation process for each extract experienced changes in the profile of secondary metabolites, starting from UV absorption, spot intensity, compound levels, types of compounds contained, and their antioxidant activity.*

KEYWORDS*: Profile, Antioxidant, Tagetes erecta, Clitoria ternatea, LC MS/MS*

INTRODUCTION

The diversity of medicinal plants has been known since ancient times, and studies related to the benefits of plants as medicine continue to develop today. One of the provinces in Indonesia, namely the Province of Bali, has medicinal plants that have been passed down from generation to generation. People in Bali use "Usada" as the basis for natural herbal medicine. The efficacy of plants in the therapy is supported by their metabolites, such as alkaloids, lignins and lignans, terpenoids, steroids, and flavonoids (Croteau, et al., 2000). The plants that will be further identified for their metabolite profiles are the marigold flower (*Tagetes erecta* L.) and the butterfly pea flower (*Clitoria*

ternatea L.).

According to research (Ingkasupart, et al., 2015), the main ingredients found in marigold flowers are flavonoids and carotenoids. Carotenoids (Lutein) are the primary pigments in marigold flowers. Flavonoids are secondary metabolites that provide pharmacological effects in their activity as antioxidants. Antioxidants are electron donors or reducing agents and can break the chain reaction of free radicals. Antioxidants can disable the development of oxidation reactions by preventing the formation of radicals. Antioxidants can inhibit oxidation reactions by binding to free radicals and highly reactive molecules. Antioxidants can be sourced from catalase enzymes, vitamin A, β-carotene, vitamin C, and vitamin E, as well as compounds from plants such as flavonoids, phenolics, and carotenoids). grapes from the Fabaceae family. The main ingredients reported are taraxerone and taraxerol. There are also tannins, steroids, flavonoids, saponins, triterpenoids, and anthocyanins. (Jamil, et al., 2018)

The Metabolite profile is a method that used to identify both qualitatively and quantitatively the metabolite compounds contained in plants (Ellis, et al., 2007). The increase in the profile of secondary metabolites in plants can occur due to fermentation. Identifying secondary metabolite profiles from the fermentation process of marigold and butterfly pea flower extracts using spectro-photodensitometry and LC-MS/MS will provide qualitative information regarding chromatographic patterns, molecular weight, retention time, and compound structure. This study aims to identify the secondary metabolite profiles of the control and fermented extracts of butterfly pea and marigold flowers, supported by studying the activity of the extracts by testing the antioxidant activity using the DPPH method.

MATERIAL AND METHODS

Chemicals and Instrument

The instrument used is analytical scales, ovens, a simplicia grinding machine, a set of percolation tools, water bath, silica gel GF60, spatula, stirring rod, vial, drip pipette, measuring pipette, funnel, round flask, beaker (Pyrex), test tube, measuring cup, Erlenmeyer flask (Pyrex), the crucible, measuring flask, ultraviolet lamp (Camag), UV visible spectrophotometer (Thermo Scientific), cuvette, crutches, measuring flask, thin layer chromatography vessel, and micropipette (Thermo Scientific).

The main sample used in this study were marigold flower simplicia (*Tagetes erecta* L.) and butterfly pea flowers (*Clitoria ternatea* L.) obtained from a traditional market in Denpasar, Bali. The materials used in this study were aqua dest, n-hexane, ethyl acetate $C_4H_8O_2$, methanol CH_3OH, hot water, FeCl3, HCl 37%, boric acid P, chloroform, sulfuric acid 97%, HCl 2 N, Bouchardat

reagent (2 % iodine in a 4 % solution of potassium iodide), Mayer reagent (mercuric chloride 1.36 g and of potassium iodide 5.00 g in water 100.0 ml, oxalic acid P, anhydrous acetic acid, Petrolium ether, acetone, amyl alcohol, and chloroform, filter paper, cotton, aluminum foil, parchment paper, sodium carbonate, Folin-Ciocalteu reagent, aluminum (III) ascorbic chloride acid, quercetin, reagent 2,2- diphenyl-1-picrilhydrazyl (DPPH).

Extraction

One g of marigold flower simplicia powder was extracted using n-hexane, and butterfly pea flower simplicia powder using methanol using the percolation method. It was sonicated for 15 minutes and then put into a 50 mL burette for filtering. The filtrate is accommodated with a measuring flask until 25 mL

Phytochemical Screening

Phytochemical screening was carried out to determine the content qualitatively of secondary metabolites in the extract. Phytochemical screening includes flavonoids, tannins, steroids/triterpenoids, alkaloids, and saponins.

Alkaloids

A total of one g of extract was added to 5 mL of 25% ammonia and stirred, then 20 mL of chloroform was added and firmly ground and filtered. The filtrate is extracted with HCl 2 N. Water phase is fed into two tubes. In the first test tube, add a few drops of Mayer reagent. The appearance of a white precipitate indicates a positive test result. And in the second test tube, a few drops of Dragendroff reagent were added, and the appearance of the red color of the solution/precipitation indicated the presence of alkaloids (Farnsworth, 1966)

Flavonoids

One g of the extract was boiled with 50 mL of distilled water for 15 minutes and filtered. This extract solution was tested for flavonoids, phenols, quinones, saponins, and tannins. A total of five mL of filtrate was put into the test tube. Some magnesium powder, 1 mL of concentrated HCl, and 5 mL of amyl alcohol were added and then gently shaken and allowed to separate. The appearance of red, brick-red, and purplish-red colors in the amyl alcohol layer indicates the presence of flavonoid group compounds (Farnsworth, 1966)

Saponins

5 mL of extract solution is put into the test tube and vigorously shaken vertically for 10 seconds until a stable foam 1 – 10 cm high is formed 10 minutes. The sample is indicated to contain saponins if the resulting foam does not disappear and remains stable with the addition of a few drops of HCl 2 N (Depkes RI, 1995)

Phenols

Five mL of extract solution is put into the test tube, and then a few drops of 1% FeCl3 solution are added. The result is positive for

phenol if blue, blue-black, green, and blue-green solution or precipitation appear (Farnsworth, 1966)

Tannins

Five mL of the extract solution was put into each of the 3 test tubes. The first test tube was added one mL with 5% FeCl3, the second test tube was added with 1% gelatin, and the third test tube was added with the reagent (2 mL 40% formaldehyde and 1 mL HCl P 37%), then was boiled. The first test tube indicated positive tannins if the blue, blue-black, green, and blue-green solution/precipitation appear. The second test tube indicated positive tannins if a white precipitate appeared. The third test tube showed positive tannins if a pink precipitate was formed (Saito, 2001)

Steroids/Triterpenoids

One g of the extract is put into a mortar and added 20 mL of ether and stirred, then filtered and evaporated into an evaporative dish. The residue on the evaporating dish is added two drops of acetic anhydride and one drop of sulfuric acid 97% (Liebermann-Burchard). The appereance of blue or blue-green indicates the presence of the steroid group, and red, pink, or purple indicates the presence of the triterpenoid group (Farnsworth, 1966).

Profiling Secondary Metabolites with TLC-Photodensitometry

TLC profile monitoring was carried out using silica gel GF254 stationary phase. The mobile phase used for the n-hexane extract of marigold flowers was n-hexane: ethyl acetate (15:2). The methanol extract of butterfly pea flower used the mobile phase ethyl acetate: glacial acetic acid: formic acid: water (9:1:1:1). The spray reagent used was 10% H2SO4. The plate size used is 7 x 10 cm with a top and bottom limit of 1 cm. The extract with a concentration of 1g/50mL was bottled 5 µL on the TLC plate, then put into a chamber filled with saturated mobile phase, then identified the spots under UV light at 254 nm and 366 nm. The plates observed under UV light were scanned with TLC densitometry At 450 nm for the marigold flower and 366 nm for the butterfly pea flower. The long-wavelength extract of the butterfly pea flower was measured at 366nm. The measurement data results that have been processed will be displayed on the computer. Rf values, area (AUC), chromatograms, and UV spectra were recorded from the TLC Photodensitometry Total phytochemical content

Total phenolic content

The total phenol content in the extract was measured using the Folin-ciocalteu reagent (Pourmorad, et al., 2006). The total phenol content in the extract was measured using the Folin-ciocalteu reagent and Gallic acid as the standard (Pourmorad, et al., 2006). Gallic

acid was dissolve in ethanol to make series concentration 60 - 110 mg/L was reacted with 500 µL of 10% Folin-Ciocalteu. 400 µL Na2CO3 1M is added, forming a colored complex compound. The solution was incubated for 30 minutes. Absorbance is measured at 765 nm. The standard curve equation created with the x-axis indicates the concentration of the gallic acid, and the y-axis indicates the absorbance.

The determination of the level is carried out by making a solution of the test sample. The concentration of marigold control and fermentation extract solutions was successively 10,000 µg/mL and 5,000 µg/mL. In comparison, the concentration of the control extract solution and butterfly pea fermentation was 10,000 µg/mL and 5,000 µg/mL. 50µL of each test solution was taken, and 10% Folin-Ciocalteu reagent 0,5 mL was added. Leave it on for 5 minutes, then add 0,4 mL Na2CO3. This mixture would form a colored complex compound. Then the compound is measured for absorbance using a UV-Vis (Thermo scientific) Spectrophotometer at a wavelength of 765 nm. The absorbance data is entered into the standard curve equation. The yield data is expressed as mg/100 g gallic acid equivalent in milligrams per 100 gram (mg GAE/100 g) of dry extract. Measurements are made in 6 repetitions

Total flavonoid content

Measurement of total flavonoids was carried out using the colorimetric method with quercetin as standard (Chang et al., 2002). The quercetin concentration range from 60-100 µg/mL was prepared from dilutions of 500 µg/mL stock solutions. Each quercetin solution was taken in an amount of 100 µL and then added 300 µL methanol p.a, 20 µL of 10% AlCl3, 20 µL of 1M sodium acetate, and 560 µL of distilled water, replicated three times. All mixtures are incubated for 30 minutes. The extract preparation uses a p.a methanol solvent with 3x replication. Absorbance was measured using a UV-Vis (Thermo scientific) Spectrophotometer at 415 nm. Total flavonoids were calculated using a quercetin calibration curve as g quercetin equivalent per 100 g extract (g QE/100 g). Total flavonoid levels of fermented and control extracts on marigold and butterfly pea flowers were compared using the same procedure as quercetin. The concentrations of the control and fermented marigold flower extracts were 5000, while the concentrations of the control and fermented butterfly pea flower extracts were 10,000 and 4,000, respectively. The absorbance data is entered into the standard curve equation. Yield data are expressed as the g of equivalent quercetin equivalents per 100 g of extract (g QE/100 g extract).

Total carotenoids content

Marigold flower Simplicia powder (fermented and controlled) as much as 5 grams, extracted with 50 mL acetone. Stir and then filter. Tests were carried out using

UV-Vis spectrophotometry (Thermo Scientific). The filtrate is taken 1 mL and put into the cuvette. The absorbance was measured at a wavelength of 470nm, 645nm, and 663nm on a spectrophotometer to calculate the amount of chlorophyll A and B first. Of the carotenoids in plants, the association with green pigments requires prior analysis of chlorophyll A and B from the formula by the formula. (Pallabi Kundu, 2016)

$$\text{chlorophyll a}=[12.7(A663)-2.69(A645)] \times V/(1000 \times W)$$

$$\text{chlorophyll b}=[22.9(A645)-4.68(A663)] \times V/(1000 \times W)$$

$$\text{Total Carotenoid (mg/g)} =[1000(A470)+3.27\{(\text{ chlorophyll a})-(\text{ chlorophyll b})\}] \times V/(W \times 229)$$

Description: A (Absorbance); V (Volume of acetone); W (simplicia

Antioxidant Activities

DPPH radical scavenging activity

Preparation of DPPH stock solution by weighing 2.5 mg of DPPH powder and then adding 50 mL of methanol p.a to obtain a 50 ppm DPPH mother standard solution. The absorbance of DPPH was measured at a maximum wavelength of 517 nm. The DPPH absorbance measurement was carried out by pipetting 2 mL of the DPPH standard solution, then placing it in a cuvette, and observing the absorbance on a UV-Vis spectrophotometer. In the IC_{50} antioxidant activity test, concentration variations were made from the stock solution on the fermented extract and marigold flower control (40ppm - 90ppm). Variation of concentration of fermented eggplant extract (40 ppm - 100 ppm) and butterfly pea flower control (40 ppm - 90 ppm). To each 0.5 mL of sample with a different concentration, 0.5 mL of DPPH solution (1:1) was added and incubated for ± 30 minutes in a brown vial. Each test was repeated three times. The absorbance of each sample was measured at a wavelength of 517 nm. The IC_{50} value is calculated using the equation so that the levels of each sample can be obtained.

Identification of Secondary Metabolites of Fermented Extract and Control of Butterfly Pea Flower Using LC MS/MS

1.5 mg methanol extract of fermented and butterfly pea flower control, added with methanol (LiChrosolv, Hypergrade for LC-MS, Merck KGaA, Darmstadt, Germany) and sonicated for 10 min until completely dissolved. The samples were then filtered through a 0.22 µm PTFE syringe Filter (Waters, Milford, Massachusetts, USA) to obtain samples with a final concentration of 1 mg/mL. The LC-MS analysis was measured on Waters Xevo-G2 XS QtoF

using Waters BEH C18 column 1.8 µm (50 mm) in MSn positive, sensitive polarity mode. The solvents were acetonitrile (B) and water (A) supplemented with 0.1% formic acid. Starting gradient from 5% B, hold for 1 min and increase gradually to 100% B in 10 min, hold at 100% for 3 minutes and bring back to initial gradient for 3 min to equilibrate the column with a total run of 17 min and the flow rate of 0.3 mL/min. Each run was compared to a blank sample, and the injection volume was 1 µL. The measurements were analyzed using UNIFI software version 1.5, and the peaks were tentatively assigned with the comparison to the Waters built-in library. MS conditions were as follows: column temperature 40 °C, mass range: 100–1200 Da, cone voltage 30 V, capillary 2kV, source temperature 120°C, desolvation temperature 500°C, cone gas flow 50 L/h, desolvation gas flow 1000 L/h, collision energy (ramp: 10–40 eV). Leucine enkephaline was used as an internal mass correction, infused every 10s during the whole run (Dewi, et al., 2022)

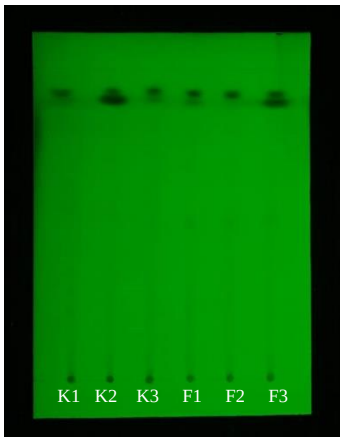
RESULTS AND DISCUSSION

Extraction using percolation method. This method was chosen because it is more efficient than the maceration process. After all, the saturated solvent will be replaced by a new solvent. The solvents used for extraction were n-hexane and methanol for each sample. The solvent choice is based on each sample's physical and chemical properties (Zhang, et al., 2018). From the results of extraction by percolation, the extract yield was obtained from the n-hexane extract of the marigold flower control and the methanol extract of the butterfly pea flower control, respectively 13.16% and 9.39%. Phytochemical screening showed that in the control and fermented marigold flowers, the fermented and the control samples showed the same results, containing flavonoids, phenols, and steroids or triterpenoids. Based on previous studies, the simplicia of marigold flowers contains flavonoids, steroidal/triterpenoid alkaloids, and phenolics (Pramitha, et al., 2018). Research by Kumar & Dwivedi, (2017) conducted a screening but did not detect the saponin content in the hexane extract of the marigold flower. (Cahyaningsih, et al., 2019) found that butterfly pea flowers contain flavonoids, phenols, saponins, and steroids/triterpenoids, but no alkaloid compounds were detected. The results of the phytochemical screening are presented in Table 1.

Table 1. Phytochemical Screening of n-hexane extract marigold flowers and methanol extract butterfly pea flowers

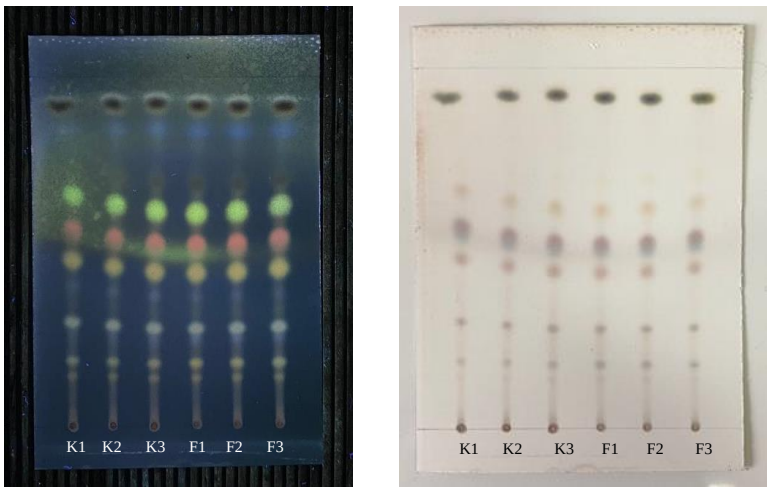
Secondary Metabolite Group	**Plant Material**			
	Marigold Control	Marigold Fermentation	Butterfly pea Control	Butterfly pea Fermentation
Flavonoids	+	+	+	+
Tannins	-	-	-	-
Phenols	+	+	+	+
Quinon	-	-	-	-
Saponins	-	-	-	-
Steroids/triterpenoids	+	+	+	+
Alkaloids	-	-	-	-

The control and fermented marigold flower extracts had the same separation pattern under UV 366 nm before and after spraying with 10% H_2SO_4 reagent. After being sprayed with 10% H_2SO_4 reagent, the compounds in the sample did not show any spots or different intensity of spots between control and fermentation when viewed from the separation by TLC. As shown in the figure 1



(a)

(b)



(c) **(d)**

Figure 1. TLC Chromatogram of marigold flower n-hexane extract with stationary phase silica gel GF_{254}, observed under (A) UV light 254nm, (B) UV light 366nm, (C) UV light 366nm after sprayed by 10% H_2SO_4, (D) Visible Light after sprayed by 10% H_2SO_4, (KI-K3) marigold flower extract control, (F1-F3) marigold extract fermentation.

The AUC value showed an increase and decrease that did not differ much between the fermented and control extracts. Eleven peaks appeared in the control extract, but only ten peaks appeared in the fermented. The variations in the peaks formed were due to the content of secondary metabolites contained in the n-hexane extract of marigold flowers, such as the detection of flavonoids, phenols, quinones, and triterpenes. The main content reported is carotenoid pigments (lutein and zeaxanthin) from the non-polar triterpenoid group (Ingkasupart dkk., 2015). The chromatogram pattern, Rf, and AUC (area under the curve) values can be seen in Figure 2 below

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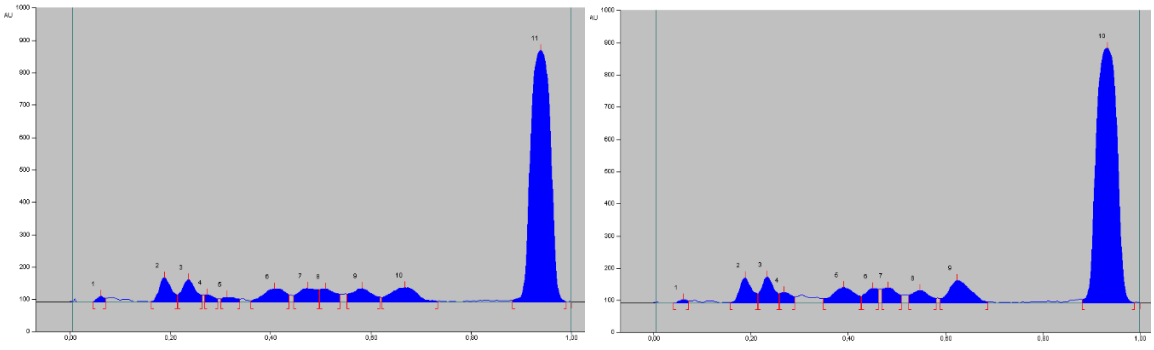


(A) (B)

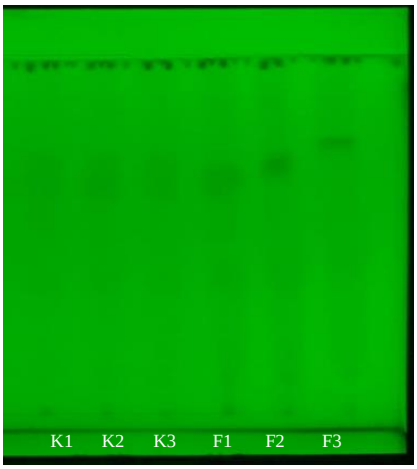
Figure 2. TLC Densitometry Chromatogram of Marigold Flower Extract, (A) Fermentation Extract, (B) Control Extract

After being visually viewed with UV at a wavelength of 254 nm and 366 nm, the plates were detected using TLC densitometry. The following Rf and AUC (Area Under Curve) values are in Table 2 below.

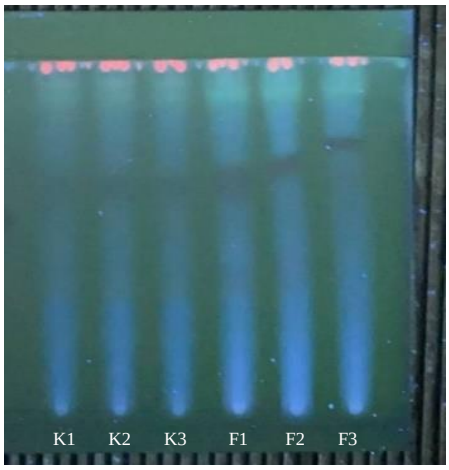
Table 2. The Result of Rf and AUC Values

Rf Control	AUC Control	Rf Fermentation	AUC Fermentation
0,06	153,1	0,06	234,4
0,19	1783,3	0,19	1625,1
0,23	1810,4	0,24	1677,4
0,27	705,4	0,27	392,6
0,48	1286,6	0,47	1468,2
0,55	1289,0	0,58	1556,2
0,93	29919,9	0,94	27173,1

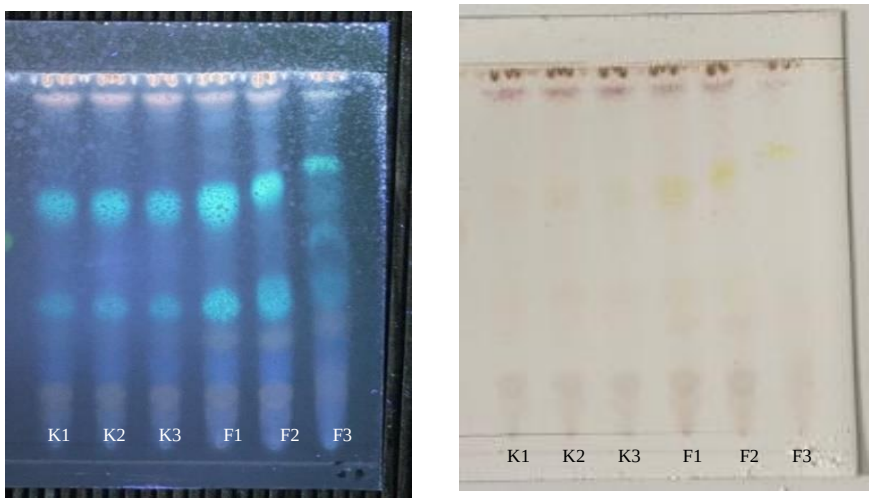
In contrast to the chromatogram profile of the butterfly pea flower, the TLC profile of the fermented extract produced thicker spots compared to the control. More spots appeared when viewed under 366 nm UV light after being sprayed with 10% H2SO4 reagent, supported by peaks in densitometry showed four peaks in the fermented extract and only four peaks in control, indicating the formation of new compounds during the fermentation process, supported by the fact that the AUC value of the fermented extract at the same Rf as the control increased.



(a)



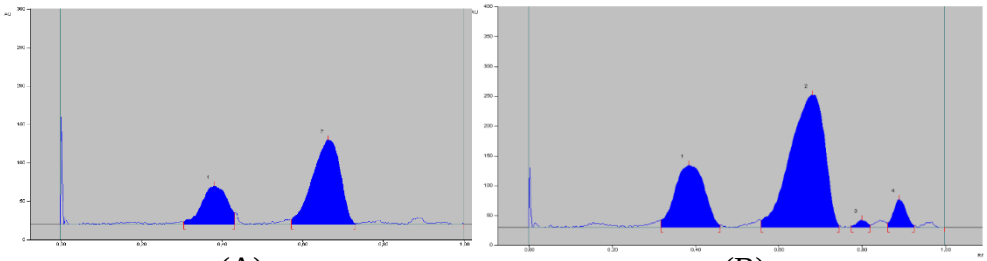
(b)



(c) **(d)**

Figure 3. TLC Chromatogram profile of butterfly pea flower methanol extract with stationary phase silica gel GF_{254}, observed under (a) UV light 254nm, (b) UV light 366nm, (c) UV light 366nm after sprayed by 10% H_2SO_4 (d) Visible Light After sprayed by 10% H_2SO_4, (KI-K3) control of butterfly pea flower extract, (F1-F3) fermentation of butterfly pea extract and

The result means that the compounds formed during the fermentation process are high. The secondary metabolite chromatogram profile can be used to evaluate the uniformity of the contents in these plants, in addition to visually observing the extract's stability. Butterfly pea flowers are reported to contain primary secondary metabolites, namely anthocyanins from the flavonoid group (Handito, et al., 2022). TLC densitometry results, chromatogram patterns, Rf values, and AUC (area under the curve) after visual observation with UV can be seen in the image below



(A) (B)

Figure 4. TLC Chromatogram of Butterfly pea Flower Extract, (A) Control Extract, (B) Fermentation Extract

In line with the results of increasing the intensity of the spots on the chromatographic pattern, the acquisition of the AUC value of the fermented extract has increased. This means that the levels of compounds formed during the fermentation process are higher. The chromatogram fermentation process produces four peaks indicating new compound formation. The Rf and AUC

values for the control and fermentation extracts can be seen in the table below

Table 3. The Rf and AUC values for the control and fermentation extracts

Rf Control	AUC Control	Rf Fermentation	AUC Fermentation
0,38	2811,8	0,38	6580,9
0,68	6774,6	0,68	16337,5

The total phenolic content of the extract was determined using the Folin-Ciocalteu method. Gallic acid was used as the standard phenolic compound. The mixture was incubated for 30 minutes, and the absorbance was measured at a wavelength of 765 nm. The results are presented in the table below.

Table 4. Total Phenol Content from Marigold Flower N-Hexane Extract and Butterfly pea Flower Methanol Extract

Extract	**Total Phenol Content (g GAE/ 100g)**
Fermentation Butterfly pea	0,900± 0,037
Control Butterfly pea	0,816 ± 0,024
Fermentation Marigold	1,154 ± 0,066
Control Marigold	1,007 ± 0,044

Results are expressed as means ± SD replicated 6 times Standard deviation, GAE: Gallic acid equivalent.

The results of the total phenol test showed that the fermented extract had a higher total phenol content. The aerobic fermentation process increases phenolic content because, during the fermentation process, enzymes are released from the natural microorganisms sampled during fermentation which are available in the form of chemical compounds such as flavonoids, tannins, and phenylpropanoids (Messens & De Vuyst, 2002). In addition, complex polyphenol compounds will be hydrolyzed in the fermentation process to become more direct and active. Phenolics and flavonoids are secondary metabolites with the most antioxidant activity in plants.

The measurement of phenol using the Folin-Ciocalteau reagent only gives a rough estimate of the total phenolic compounds present in the extract. It does not measure specific polyphenols. Furthermore, many disruptive compounds can react with reagents, thus providing increased phenolic concentrations. In addition, Tawaha et al. (2007) state that various phenolic compounds will react differently to the reagent, depending on the number of phenolic groups. The total phenol does not need to contain all the antioxidants that may be present in the extract. Phenolics and flavonoids are secondary metabolites with the highest antioxidant activity in plants. Phenolics can

stabilize and delocalize unpaired electrons in their aromatic rings. In the previous study, fermenting rice bran by Rhizopus oryzae, the phenolic acid content before fermentation was 33 mg/g extract, but there was an increase in phenolic acid compounds to 765 mg/g extract after fermentation (Schmidt, et al., 2014). Total flavonoid content was determined using a colorimetric method with aluminum chloride. Quercetin is used as a standard to create calibration curves. The flavonoid content of the extract is determined by its equivalence with quercetin standards, as shown in table below.

Table 5. Total Flavonoid Content from Marigold Flower N-Hexane Extract and Butterfly pea Flower Methanol Extract

Extract	Total Flavonoid Content (g QE/ 100g)
Fermentation Butterfly pea	0,610± 0,011
Control Butterfly pea	0,384 ± 0,041
Fermentation Marigold	0,067 ± 0,032
Control Marigold	0,418 ± 0,026

The results showed that the total flavonoid content of fermented pea methanol extract was higher than the control. The TLC densitometry spectrum profile also proved an increase in secondary metabolites in fermented extracts. The main content of the butterfly pea flower is anthocyanin which belongs to the flavonoid group. The increase in secondary metabolite content in butterfly pea flowers can be influenced by environmental factors such as UV light, light intensity, pathogen attack, drought, injury, and nutritional deficits. The optimum temperature for anthocyanin content in butterfly pea flowers is 25ºC (Ramakrishna & Ravishankar, 2011). Previous research by Srichaikul. (2018) showed that the total content of butterfly pea flower flavonoids extracted using polar solvents was 0.05 ± 0.03 µg/mg sample. Compared to this study, the levels of total flavonoids obtained from the methanol extract of butterfly pea flowers were higher. In the marigold extract, the total flavonoid content was highest in the fermented extract. The main compounds in marigold flowers are carotenoids, and temperature and light factors often affect their stability. Storage at room temperature (33-37ºC) accelerates the process of degradation of carotenoid compounds.

Measurement of antioxidant activity aims to evaluate these compounds as alternative antioxidants. Conversely, the determination of the IC_{50} value is intended to determine the concentration of antioxidant compounds that inhibit 50% of oxidation. One of the commonly used methods is DPPH (1,1-diphenyl-2-Picrylhydrazyl). Antioxidants were measured using a UV-Vis spectrophotometer (Thermo Scientific).

Testing the antioxidant activity begins with determining the maximum wavelength of DPPH at a concentration of 50 µg/ml in methanol. The maximum wavelength is 517 nm. The DPPH wavelength is usually measured in 515–520 nm (Marxen et al., 2007). DPPH compounds have a nitrogen atom with one unpaired free electron, making this DPPH compound a stable radical. Samples with antioxidant compounds will donate their hydrogen atoms to bind with nitrogen atoms from DPPH, forming non-radical DPPH molecules (DPPH-H). The principle of measuring antioxidant activity using DPPH is that there is a change in the intensity of the purple color of the DPPH radical, which is proportional to the concentration of the DPPH solution. DPPH in the radical form is purple, while DPPH in the non-radical form is yellow (Shekhar & Anju, 2014). IC_{50} values of butterfly pea and marigold flower extracts are shown in the table below.

Table 6. IC_{50} values of Marigold Flower N-Hexane Extract and Butterfly pea Flower Methanol Extract

Extract	**IC_{50} (ppm)**	**Category** (Awe dkk., 2013)
Ascorbic Acid	0,49	Very strong
Fermentation Butterfly pea	46,71	Very strong
Control Butterfly pea	75,30	Strong
Fermentation Marigold	48,30	Very strong
Control Marigold	70,67	Strong

The positive control used in testing the antioxidant activity is vitamin C to determine how strong the antioxidant potential is in the extracts of butterfly pea and marigold flowers compared to vitamin C. If the IC_{50} value of the sample is the same or close to the IC_{50} value of the positive control, then the sample has the potential to be an alternative. Powerful antioxidant. The IC_{50} value was 46.71 ppm, while the fermented marigold flower extract had an IC_{50} value of 48.30 ppm. Both fermented extracts showed intense antioxidant activity with the IC_{50} category <50 ppm. They were supported by research by Kusmiati et al. (2018) tested the antioxidant activity of marigold flowers and obtained an IC_{50} value of 50.641 µg/mL, which means that the antioxidant activity is intense. Research Niranjan et al. (2020) tested the antioxidant activity of the methanol extract of butterfly pea flowers to produce an IC_{50} value of 92.42 µg/mL, so the activity was classified as strong. The lower the IC_{50} value, the higher the antioxidant capacity produced (Molyneux, 2004).

The butterfly pea flower metabolite profile was analyzed using LC-HRMS, and the LC was connected to a mass spectrophotometer with an ESI (+) ion source. The MS analyzer used is Xevo G2 XS-QToF with UNIFI software. Analysis was performed by sample injection. The injected sample will enter the column. Inside the column, the samples will be separated based on their polarity. The stationary phase was column C18, while the eluent used was acetonitrile (B) and water (A) plus 0.1% formic acid with a gradient system. A mixture of acetonitrile/formic acid mobile phase to speed up the separation process in the column of this system will produce polar compounds, which will be eluted first, then less polar compounds (Farag et al., 2016). The elution results will be forwarded to the mass detector (MS). In the MS system, the sample in the form of liquid will be dripped through a positively charged needle because the type of ion used is ESI (+) which the MS detector will read. The ions obtained will be separated using a Q-ToF mass analyzer. The results of the separation will appear as a chromatogram. The chromatogram results from UNIFI software can predict the molecular formula of each compound. The UNIFI software can find the mass spectrum of the compound in the sample, which matches the mass spectrum in the library. Each peak indicates one compound. The identified molecular formula must be reduced to 1 H atom, called the neutral mass, because, in the separation process, 1 H atom is added, which comes from the addition of ESI (+) ions. The table of results of LC-MS/MS analysis of methanol extract of butterfly pea flowers can be seen in the table below

Table 7. LC-MS/MS analysis of fermented butterfly pea flower methanol extract

Extract	**Fermentation**			
Clitoria ternatea	Retention Time	m/z	Compound	Molecular Formula
	3,30	595,1667	Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$
	3,65	433,1133	Kaempferol-7-O-α-L-rhamnoside	$C_{21}H_{20}O_{10}$
	4,51	447,1283	Acacetin-7-galactoside	$C_{22}H_{22}O_{10}$
	6,07	285,0765	5,7-Dihydroxy-3-(4'-hydroxybenzyl) chromone	$C_{16}H_{12}O_5$
	7,85	399,3573	24-Methylcholesta-7,22dien-3β-ol	$C_{28}H_{46}O$

CONCLUSION

Chromatogram profiles of fermented extracts and marigold flower control showed increased and decreased AUC values. In contrast to the chromatographic profile of the fermented butterfly pea extract, changes occurred, such as increased spectral absorption, AUC value, thicker spot intensity on UV-Vis λ 366nm observation, more spots formed on the fermented extract, and the

peaks shown. The appearance of new compounds in fermented extracts. The secondary metabolites identified in the fermented and control extracts have varied results. The fermentation process increases the compound content and can increase its antioxidant activity, so it has the potential to be developed as a natural product-based treatment.

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