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Research Article

ISOLATION AND ANTIOXIDANT ACTIVITY OF ISOFLAVONE COMPOUNDS IN n-HEXANE EXTRACT OF HIBISCUS MANIHOT L

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A B S T R A C T

Hibiscus manihot L leaf contains isoflavone compounds that have the ability to slow down or prevent the oxidation of other molecules. The aim of this study was to isolate and determine the antioxidant capacity of isoflavone compounds contained in n-hexane extract of Hibiscus manihot L leaves associated to its ability as an anti-free radicals (free radical scavenger). Maceration of 1000 g of dry powder of Hibiscus manihot L leaves with 96% ethanol produced as much as 27.43 g viscous extract. Furthermore, the partition with n-hexane produced 4.81 g of blackish brown extract.

The results of antioxidant activity by the method of 1,1-diphenyl-2-pycrylhidrazil (DPPH) showed that n-hexane extract of Hibiscus manihot L leaves yielded IC_{50} value of 9.68 mg/mL Separation and purification of n-hexane extract were performed by thin layer chromatography and column chromatography resulted in five fractions (F_A, F_B, F_C, F_D, and F_E).

Phytochemical screening results showed that n-hexane extract contained flavonoids.The characterization of F_A isolates by spectrophotometry UV-Vis showed that F_A exhibited in two absorption bands which were at 329.60 nm (band I) and 266.30 nm (band II). It revealed that F_A contained flavonoids of isoflavone group in which no free OH groups was found on the A ring and as well as contained ortho-dihydroxy groups on the A ring. The analysis of infrared spectrum showed that F_A isolates might contain the functional groups of CH aromatic, CH aliphatic, C=O ketones, C=C aromatic and C-O-C ether. It could be concluded that F_A isolates was a flavonoid compound of isoflavone group i.e. 5,7-dihydroxy isoflavone that had strong antioxidant activity against DPPH.

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INTRODUCTION

Mamahit, *et al.* [9], reported that one of the traditional medicinal plants that has the potential to be developed as a medicinal herb is the leaf of Hibiscus manihot L, since the leaves of Hibiscus manihot L has been used to treat various diseases traditionally, such as diabetes, ulcers, heart disease, blood pressure high, osteoporosis renal impairment, seizures, and depression. Some research results show that the extract of Hibiscus manihot L leaf contains several compounds, such as flavonoids, terpenoids, alkaloids, tannins, polyphenols, saponins and serotonin. Isoflavone compounds are the derivative of flavonoids that are believed to have exogenous antioxidant activity working to increase the number of pancreatic -cells through a decrease in blood glucose and cholesterol levels in diabetic Wistar rats [1]. According to Prangdimurti *et al,* [13,15] the intake of isoflavone in suji leaf extracts which is rich in antioxidants can increase the activity of SOD and CAT in the hearts of diabetic mice, therefore it can protect cells against oxidative stress.

Isoflavone compounds are one of chemical compounds that are included in the secondary metabolites found in many plants. The compounds are generally bound or conjugated with glikon or other chemical compounds. Isoflavones can work more effective when they are free state. Free isoflavone is known as aglycone, which is very useful as a natural antioxidant. Isoflavones are found only in plants and spread throughout the parts (roots, stems, leaves, fruits). One plant that is rich in isoflavones is soybeans, in which the compounds are found the most in the endosperm and the cotyledons [5,10]. Isoflavone compounds are known to have various physiological activities including as antibacterial, anti-inflammatory, antibiotic, allergy and antioxidant [7]. Antioxidants are compounds that have the ability to inhibit oxidation caused by free radicals. Antioxidants react with free radicals to form compounds that are not reactive and unstable. The activity of isoflavone compounds as antioxidants is influenced by the hydroxy and 4-oxo groups in the isoflavones skeleton [13]. Peterson, *et al*, [11] suggested that the antioxidant activity is determined by the form of the free structure (aglycone) of a compound and the doubles OH

group, particularly on C=O at position C-3 with OH group at position C-2 or C-5. The basic structure of isoflavone compounds that have the potential to be developed as a natural antioxidant is as follows:

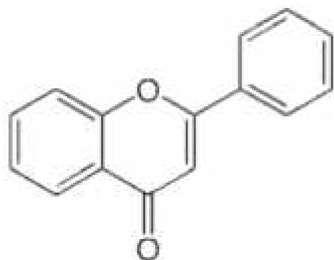

Figure 1 Basic Structure of Isoflavone Compounds

MATERIALS AND METHODS

Sample Pretreatment

The sample used in the research was the leaves of Hibiscus manihot L obtained from Ubud Gianyar Bali. Then, it was determined in Indonesian Institute of Sciences (LIPI), Unit for Plant Conservation Bali Eka Karya Botanical Garden Bedugul, Tabanan, Bali. The sample was cleaned then dried in an open space with open air circulation and was not exposed to direct sunlight. Furthermore, the dry sample was milled with a blender until it became fine powder.

Sample Extraction

One kilogram of fine powder of Hibischus Manihot L leaf was extracted by maceration using 6 L ethanol 96% for 24 hours, then the extract was evaporated using a rotary vacuum evaporator until a viscous ethanol extract was obtained. Furthermore, the viscous extract was hydrolyzed with 2N HCl for 2-3 hours. The hydrolysis result was then partitioned with n-hexane. The residue obtained was re-extracted using n-hexane. N-hexane extract was evaporated to obtain a viscous extract of n-hexane and subsequently the separation and purification were done by thin layer chromatography and column chromatography.

Phytochemical Screening of Isoflavone Compounds

Examination of isoflavone group can be done with the color reaction as follows:

1. Wilstatter Test; a certain amount of samples was added with Mg powder and concentrated HCl. Positive reaction is shown by a color changes from dark yellow to orange.
2. Bate-Smith-Matcalfe Test; a certain amount of sample was added with concentrated H_2SO_4 and heated for 15 minutes over a water bath. Positive reaction is indicated by a color change from amber to red.
3. Test with NaOH 10%; a certain amount of sample was added with a few drops of 10% NaOH. Positive reaction is shown by a color changes from dark yellow to light yellow.

Antioxidant Activity Test by 1,1-diphenyl-2-pycrylhidrazil Method (DPPH)

The antioxidant activity test of F_A isolates was done by preparing the sample solution of 1000 ppm in which 25 mg of the isolates was diluted in 25 mLvolumetric with ethanol. Then, the solutions of 1.00 mg/mL, 2.00 mg/mL, 3.00 mg/mL, 4.00 mg/mL and 5.00 mg/mL were made. 1.0 mL of each solution was taken by pipette and then they were put in different test tubes followed by the addition of 1.0 mL of 1,1-diphenyl-2-pycrylhidrazyl of 0.004% and 2.0 mL ethanol. The mixture was allowed to stand for 30 minutes, and then the absorbance was measured with UV-Vis spectrophotometer at a wavelength of 517 nm with a repetition twice. Finally, the value of IC_{50} was calculated.

Characterization of Isoflavone Compounds by Spectrophotometry UV-Vis and FTIR

The measurement of UV-Vis spectrum was performed at a wavelength of 250-500 nm. 1,0 mg of active isolates (F_A) was dissolved into 100 ml of methanol, then its wavelength was determined. Furthermore, the addition of shift reagent into the sample solution was done in order to determine the position of the hydroxyl group at the core of isoflavones. The FTIR spectrum measurement was carried out at the wave number area of 500-4000 cm^{-1}. The active isolates (F_A) alleged as an isoflavone group was mixed with potassium bromide (KBr). The mixture formed was placed in two containers of plate crystals of NaCl and put into an infrared spectrophotometer, and then the absorbance was measured.

RESULTS AND DISCUSSION

Extraction of Isoflavones Compounds

A total of 1000 g of Hibiscus manihot L leaves powder was extracted by maceration using ethanol 96%. The filtrate obtained was evaporated by using a rotary vacuum evaporator until all ethanol evaporated and as much as 27.43 g of a blackish brown viscous ethanol extract was obtained. Furthermore, the viscous ethanol extract was hydrolyzed with 2N HCl to separate the aglycone and glicon compounds. The hydrolysis result was repartitioned with n-hexane to obtain acid and n-hexane extracts. After that, the N-hexane extract was evaporated so that as much as 4.81 g of viscous extract of n-hexane was obtained. The antioxidant activity of the viscous n-hexane extract was ran by the *1,1-diphenyl-2-pycrylhidrazil* (DPPH) method to determine the ability in capturing free radicals as well as to see if there was a linear correlation between isoflavone content against its ability to capture free radicals. The antioxidant activity of an extract is determined by the IC_{50} (inhibition concentration). The IC_{50} values can be determined by entering the value of 50% of suppression activities into the linear regression equation by plotting the % suppression value and the test solution. The relationship between the % suppression and the IC_{50} of n-hexane extract is shown in Figure 2 and Table 1:

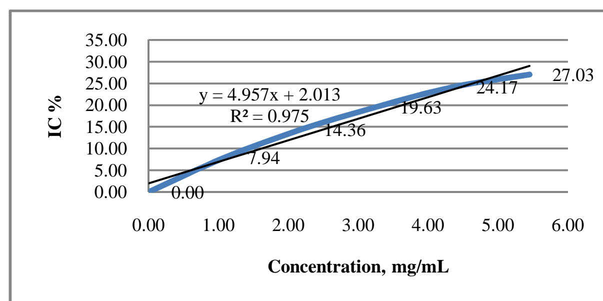

Figure 2 The relationship between the concentration of DPPH and the IC_{50} values

Table 1 Percent Suppression and IC_{50} of n-Hexane Extract of Hibiscus manihot L Leaves

Concentration (mg/mL)	Absorbance	% Suppression	Linear Regression
0.00	1.121	0.000	y = 4.957x + 2.013
1.00	1.032	7.94	$R^2 = 0.975$
2.00	0.960	14.36	IC50 = 9.68 mg/mL
3.00	0.901	19.63	
4.00	0.850	24.17	
5.00	0.818	27.03	

IC_{50} value is inversely proportional to the ability of an antioxidant compound. The smaller the IC_{50} value, the stronger the ability of a compound as an antioxidant is. The results of the IC_{50} analysis showed that n-hexane extract of Hibiscus manihot L leaves from the partition result had a very strong antioxidant activity. This was probably because of the presence of antagonistic compounds in the crude extract that were able to suppress the ability in reducing free radicals, however, fractionation with n -heksana: chloroform: ethanol 96% caused a very strong free radicals reduction which was due to the fact that the amount of flavonoids was more than that in the crude extract, consequently there was a stronger antioxidant activity. According to Sinaga (2009), a very powerful antioxidant compounds is when their IC_{50} values are less than 50 ppm and weak when they have IC_{50} values more than 500 ppm.

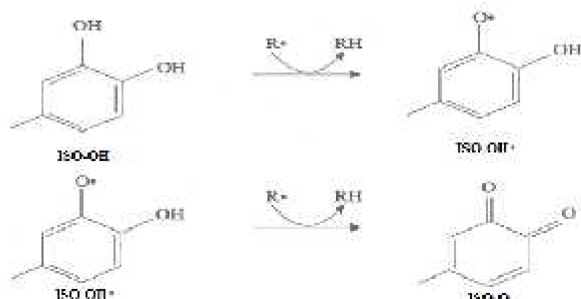

Figure 3 The mechanism of of free radicals suppression by isoflavone compounds

Separation and Purification of Isoflavone Compounds

The separation of n-hexane extract of Hibiscus manihot L leaves was performed to obtain isoflavone compound that have antioxidant activity against DPPH. The separation of n-hexane extract using column chromatography with a mobile phase of n-hexane: chloroform: ethanol (1: 1: 1) resulted in five fractions, namely F_A, F_B, F_C, F_D and F_E with different separation patterns.

Table 2 Phytochemicals Test Results of n-Hexane Extract of Hibiscus manihot L

Fraction	Rf Large	Color Test			Result
		Willstater (Mg-HCl)	Bate Smith-Metcalfe	NaOH 10%	++ Flavonoid (Isoflavon)
F_A (1-17)	0,91	Orange	Dark red	Light yellow	- Flavonoid
F_B (18-27)	0.88, 0.76	Yellow	Blue	Orange	- Flavonoid
F_C (28-42)	0.88, 0.79, 0.71	Light brown	Light brown	Black	- Flavonoid
F_D (43-64)	0.86, 0.71, 0.59	Yellow	Green	Green	- Flavonoid
F_E (65-96)	0.72, 0.64	Light yellow	Clear	Clear	++ Flavonoid (Isoflavon)

Furthermore, the phytochemical test results showed that F_A fraction was found to positively contain flavonoids of isoflavone group with a typical color intensity and it was relatively pure thin-layer chromatographically with different Rf value. The phytochemical test results are presented in Table 2.

Of the five fractions obtained, only F_A fraction positively contained flavonoids of isoflavone group, this was because at the addition of the color reagent it showed a typical color change for flavonoids. After that, the purity test was carried out by thin layer chromatography. This test resulted in a chromatogram showing a single spot suggesting that FA was relatively pure thin-layer chromatographically and further, the F_A isolates was identified to determine the class of isoflavone compounds.

Characterization of the Compound of Isolation Result UV-Vis spectrum analysis

The results of the analysis of isolates F_A with UV-Vis spectrophotometer is presented in Figure 4, while the wavelength and absorbance are shown in Table 3.

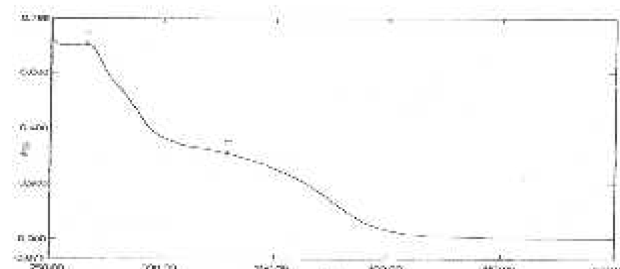

Figure 4 UV-Vis spectra of F_A Isolates

Table 3 UV-Vis spectra data of F_A Isolates

Isolate F_A	Wavelength (nm)	Absorbance
Band I	329,60	0,312
Band II	266,30	0,705

From the UV-Vis spectrum of isolates F_A in methanol it was observed two absorption bands, i.e. band I was at a wavelength of 329.60 nm and band II was at a wavelength of 266.30 nm. The spectrum of isolates F_A provided an absorption range of 245-275 nm in bands I and an absorption range of 310-330 nm on band II, showing the absorption ranges of flavonoids of isoflavone class [10]. The position of the hydroxy group at the core of flavonoids was determined by the addition of a shift reagent. The absorption of band II effected the hydroxylation of A ring, while the absorption band I affected the hydroxylation of ring B and C. Hydroxylation is influenced by bathochromic shifting, whereas methylation and glycosylation will cause a shift in the band to the lower wavelength (hipsochromic). The results of the absorption shifts after the addition of shift reagent can be seen in Table 4.

Table 4 Isolates F_A Absorption Shift with the Addition of the Shift Reagent

Reagent slide	(nm)		Friction (nm)	
	Band I	Band II	Band I	Band II
MeOH	266,30	329,60	-	-
MeOH + NaOH	268,70	390,01	+64,5	+3,7
MeOH + $AlCl_3$	273,10	339,30	+9,6	+8
MeOH + $AlCl_3$ + HCl	276,70	338,02	+8,2	+12,5
MeOH + NaOAc	270,40	398,50	+68,8	+4,9
MeOH + NaOAc + H_3BO_3	265,01	347,10	+16,7	+2,3

After the addition of NaOH shift reagent, F_A isolates exhibited a bathochromic shift in the band I of 64.5 nm and in the band II of 3.7 nm as ell as a decrease in the absorption strength. This showed that the F_A isolates contained isoflavone compounds, in which the A ring indicated the presence of an OH group at position 5 [10]. The addition of aluminum chloride ($AlCl_3$) as shift reagents caused the formation of complexes with ortho-hydroxy groups or hydroxy ketone resulting in a bathochromic shift of 8 nm on the second band. Meanwhile, with the addition of hydrochloric acid (HCl) resulted in complex redecomposition due to the presence of unstable Al formed on ortho-hydroxy groups, resulting in a bathochromic shift of 12.5 nm which indicated the presence of hydroxy group on ring A in C-5 [10]. The addition of shift reagents NaOAc ionized the most acid resistant hydroxyl group of isoflavone i.e. 7-OH causing a bathochromic shift in the band II at 4.9 nm, while the addition of the shift reagent NaOAc + H_3BO_3 caused a hypsochromic shift in the band II at 2.3 nm indicating the absence of ortho-hydroxy groups on the ring A (5,7).

Infrared spectrophotometer Analysis

The results of the infrared spectrum of F_A isolates is presented in Figure 8 and Table 5

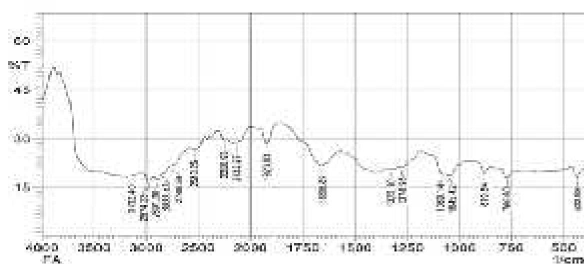

Figure 5 Infrared spectra of F_A isolates

Table 5 Interpretation of Infrared Spectra of F_A Isolates

Wavenumber isolate F_A	Wavenumber (theory)	Shape ribbon	Intensity	Placement group
3132,40	3000-3450	Splay	Moderate	C-H aromatic
2974,23	2700-3000	Sharp	Weak	C-H aliphatic
1656,85	1650-1900	Sharp	Very strong	C=O
1465,90	1400-1500	Low	Moderate	C=C aromatic
1325,10	1300-1400	Low	Strong	C-H aliphatic
1274,95	800-1300	Splay	Very strong	C-O
879,54	650-1000	Sharp	Moderate	C=C
769,80	700-900	Sharp	Weak	C-H aromatic

The infrared spectra showed a widening absorption and weak intensity at the wave number of 3132.40 cm^{-1} which was alleged to be the sorption of C-H aromatic. This suspicion was strengthened with the presence of their absorption at the wave number region of 1325.10 cm^{-}1 and 769.80 cm^{-1} which indicated the presence of aliphatic C-H and C-H aromatic. The spectrum results appeared at the the wave number of 2974.23 cm^{-1}, 2891.30 cm^{-1}, 2833.43 $cm^{-1}$and 2748.56 cm^{-1} in the form of widening bands and moderate intensity showed the presence of aliphatic C-H group. Absorption bands in the wave number area of 1656.85 cm^{-1} of moderate intensity was the typical of C=O ketones. The presence of sharp absorption and moderate intensity at 879.54 cm^{-1} revealed the C=C aromatic. Absorption with moderate band at 1274.95 cm^{-1} and 1080.14 cm^{-1} revealed the presence of C-O-C ether. In the infrared spectrum there was no OH group observed. This was caused by the formation of hydrogen bonds between the OH group that takes adjacent positions (ortho position), so that the spectrum revealed that the F_A isolates had the functional groups of C-H aromatic, C-H aliphatic, C=O, C=C and C-O.

From the data of UV-Vis spectrophotometry, FTIR and shift reagents, therefore the flavonoid compounds found in F_A isolates was a flavonoid compound of isoflavone classes with no free OH groups in A ring but contained o-diOH in A ring. Rimbach [12] has reported that isoflavones are able to neutralize free radicals and are able to modulate intracellular antioxidant superoxide dismutase. Figure 6 is the prediction of the structure of 5,7-dihydroxy isoflavone :

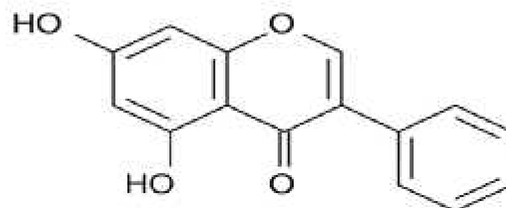

Figure 6 The Structure of 5,7-dihydroxy isoflavone

CONCLUSIONS

1. The F_A isolates of n-hexane of Hibiscus manihot L leaves contained isoflavone compounds with a wavelength of 329.60 nm and 266.30 nm, had functional groups of CH aromatic, aliphatic CH, C=O, C=C aromatic, and C-O with no free OH groups in the A ring and had o-diOH group in the ring A.
2. Isoflavone compounds contained in the extracts of n-hexane of Hibiscus manihot L leaves had naturally antioxidant activity with suppression percentage of 87.03 ppm and IC_{50} of 9,68 mg/mL, therefore it is potential in preventing oxidative stress due to the formation of free radicals.

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