ISOLATION AND IDENTIFICATION OF ANTIOXIDANT COMPOUNDS FROM PIPER METHYSTICUM ROOT

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Abstract - Bioassay-guided fractionation of the ethyl acetate extract of kava root resulted in the isolation and purification of six fractions using column chromatography under direction of DPPH and ABTS assays. They included three flavonoids: 5-hydroxy-4',7-dimethoxyflavanone (C1), matteucinol (C2) and 5,6-dihydroxy-7-methoxyflavone (C3) and three kavalactones: 5,6-dehydrokawain (DK) and 5,6-dihydromethysticin (C4), DK (C5), and yangonin (C6). The evaluation of free radical scavenging activity of the isolated substances by DPPH and ABTS assays revealed that C1 (IC_{50}: ABTS = 203.5 µg/ml), C2 (IC_{50}: ABTS = 173.1 µg/ml), C3 (IC_{50}: ABTS = 75.2; DPPH = 75.1 µg/ml), and C4 (IC_{50}: ABTS = 181.7; DPPH = 1210.2 µg/ml) have significant antioxidant properties. This study was the first to purify and identify the flavonoid C3 in kava root and report on its antioxidant potential.

Index Terms - Piper methysticum L., antioxidant activity, antibacterial activity, flavonoid, kavalactone.

I. INTRODUCTION

Kava (Piper methysticum), is an exclusive perennial plant found on the Pacific Islands. Kava root has traditionally been used by indigenous people as an effective folk remedy for a variety of diseases, such as gonorrhea, menstrual pain, tuberculosis, arthritic conditions, and chronic gout (Meyer et al., 1979; Lebot et al., 1992). Our previous work concerning the antioxidant activity of kava root was conducted on different extracts.

The results indicated that acetone extract exhibited the strongest radical scavenging property (Xuan et al., 2008). However, the compounds responsible for the antioxidant ability in the crude extract have not been previously investigated so far, except for the sporadic evaluations about the antioxidant capacity of several kavalactones (Wu et al., 2002; Chompoo et al., 2012). In this study, an activity-guided trial was proposed to isolate and purify the bioactive compounds using column chromatography (CC). The antioxidant activity of extracts and compounds were examined by in vitro DPPH and ABTS assays.

II. MATERIALS AND METHODOLOGY

A.Plant Material

The dried root part of commercial kava plant was obtained from Kava King Products, Inc (Orchard St, Ormond Beach, USA) on 2 May 2017. The powder sample was stored at -20 ◦C in the freezer prior to experiments.

B.Preparation of Extract and Fractions

The extraction of kava root was conducted as described previously (Van et al., 2018). An amount of 907.2 g of the dried kava root powder was immersed in 3 L of acetone for two weeks at room temperature. The extract was filtered, and then evaporated to dryness using a rotary evaporator (SB-350-EYELA, Tokyo, Japan) at 45 ˚C. A small amount of the acetone extract (1.5 g) was diluted with methanol to obtain various concentrations 500, 1000, 2000 ppm for further bioassays.

The rest part of the crude extract (47.89 g) was suspended in distilled water (100 mL), and successively partitioned with hexane (H), chloroform (C), and ethyl acetate (EtOAc) to give the corresponding extracts (0.42 g (0.9 %), 46.86 g (99.94 %), 2.6 g (5.54 %), 0.34 g (0.73%), respectively). The EtOAc fraction showed the strongest radical scavenging capacity was selected for further isolation.

C.Isolation and Purification Procedure of Antioxidant Compounds

The isolation process of bioactive compounds in kava root was reported by Van et al., 2018. The ground kava root (165.9 g) was first immersed in acetone-acetonitrile (4:1) at room temperature for one week. The obtained extract was then filtered through a filter paper (Whatman No.5C, 110 mm) until the powder sample has all been removed, the resulting solvent was evaporated using a rotary evaporator (SB–350–EYELA, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 45 °C to yield 9.4g crude extract. After that, the crude extract was dissolved in distilled water, then extracted successively with hexane and EtOAc.

The crude extract (6.5 g) obtained by evaporating the EtOAc layer was further chromatographed into a column ((20 mm diameter × 500 mm height, Climbing G2, Tokyo, Japan) over a silica gel (size Å 60, 200-400 mesh particle size, Sigma-Aldrich) and eluted with a gradient of n-hexane-EtOAc (10:0-0:10

v/v). The fractions were collected separately (frs.; each of 100mL), and concentrated under a rotary evaporator, followed by inspection of thin layer chromatography (TLC) to verify the purity of compounds and determine the number of components.

The recorded similar spots on TLC sheets were combined. Fractions were then crystallized at room temperature to give six isolated compounds and mixtures as described in Figure 1. Compounds C1, C2, C3, and C5 were purified after eluting by hexane, followed by rapidly filtering through a filter paper to remove the traces of impurities. The combined fractions 48-55 obtained from H:E 8:2 were solidified and dissolved in a mixture solvent of H-E 6:4 to yield a pure compound C6. The yields of compounds from C1 to C6 were 7.7, 54.7, 4.7, 28.9, 174.4, and 70.3 mg, respectively.

The chemical formulas of isolated substances were determined by Gas Chromatography–Mass Spectrometry (GC-MS) and Electrospray ionization - mass spectrometry (ESI-MS) data. The pure compounds were further subjected to ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) for elucidation of the molecular structure.

D. Identification of Antioxidative Compounds

The active compounds and mixtures were analyzed by GC-MS machine (JMS-T100 GCV, JEOL Ltd., Tokyo, Japan) equipped with a DB-5MS capillary column, 30 m in length, 0.25 mm internal diameter, and 0.25 µm in thickness (Agilent Technologies, J & W Scientific Products, Folsom, CA, USA).

The column temperature was setup initially at 50 ^{o}C without hold time, followed by an increase to 300 °C with the gradients of 10 °C/min and hold for 20 min. The temperature of injector and detector were programmed respectively at 300 °C and 320 °C, with a mass scan range 29 -800 amu. The compounds were determined by the comparison between their mass spectral fragmentation pattern with the mass spectral libraries of JEOL's GC-MS Mass Center System Version 2.65a. The compounds with the high purity were selected for further spectroscopic techniques to structure elucidation. ESI/MS analysis was implemented using mass spectrometer with electrospray ion source.

The measurements were conducted on the positive mode with an ion spray voltage of 3000V, and a capillary temperature of 350 °C. The mass spectra were recorded in full scan mode within the range 280 to 1000 m/z. The Nuclear Magnetic Resonance spectroscopy (1H (400.13 Hz) and 13C (100.612 Hz)) were recorded by a Bruker 500 model spectrometer. The pure compounds were dissolved in chloroform ($CDCl_3$) to obtain a concentration of 3000 ppm (µg/mL). The spectral characteristics of compounds were compared with those of literature to elucidate the chemical structures.

E. Antioxidant Assays

- **ABTS Radical Scavenging Assay**

The $ABTS^{+}$ radical cation was produced by reacting 15 mL aqueous solution of ABTS 7mM with 240 µL potassium persulfate 140 mL. The mixture was allowed to keep in the dark at room temperature for 16h before use. Prior to assay, the $ABTS^{+}$ was diluted with MeOH to achieve an absorbance 0.70±0.05 at 734 nm. The reaction mixture contained 1mL of prepared $ABTS^{+}$ solution and 0.12 mL of each sample was measured after incubated for 30 min in the dark at room temperature. The ABTS radical scavenging activity was expressed by the following equation:

ABTS radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

Where $Abs_{control}$ is the absorbance of ABTS radical + methanol; Abs_{sample} is the absorbance of ABTS radical + sample extract/ standard.

Based on the value of ABTS radical scavenging activity, IC_{50} value was calculated to evaluate the antioxidant ability of tested samples. The smaller value of IC_{50} is associated with the stronger antioxidant properties. The smaller value of IC_{50} is associated with the stronger antioxidant properties.

- **DPPH Radical Scavenging Assay**

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was conducted according to the method described by Minh et al., 2018 with a minor modification. The various polarity extracts were evaluated at concentrations of 500, 1000, 2000 ppm, while that of isolated compounds were 10, 50, 100, 500, 1000 ppm. A volume of 100 µL of each sample was mixed with 50 µL DPPH 0.5 nM, followed by the addition of 100 µL sodium acetate buffer (pH 5.5, 0.1 M). In this study, BHT and methanol (MeOH) were used as a positive standard and control, respectively. After incubating in the dark for 30 min at room temperature, the mixture absorbance (Abs) was measured at 517 nm using a UV-VIS spectrophotometer (Multiskan GO; Thermo Fisher Scientific, Finland). This assay was implemented in triplicate and the antioxidant activity was calculated as the following equation:

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$

Where $Abs_{control}$ is the absorbance of control reaction, and Abs_{sample} is the absorbance of the test substance.

Based on the value of DPPH radical scavenging activity, IC_{50} value was calculated to evaluate the antioxidant ability of tested samples. The smaller value of IC_{50} is associated with the stronger antioxidant properties.

III. RESULTS

A. Antioxidant Activities of Crude Extracts

The various extracts of kava root were investigated for free radical scavenging capacities using ABTS and DPPH assays. The results expressed as IC_{50} values are summarized in Table 2. The IC_{50} was used as a parameter which indicates the concentration of the samples required to scavenge 50% of the free radicals.

The lower IC_{50} value represents the higher antioxidant potential. Among studied extracts, the EtOAc fraction exhibited the most effective in scavenging $ABTS^{+}$ and DPPH free radical as its IC_{50} values were 1009 and 943 µg/mL, respectively. IC_{50} values of BHT standard evaluated by these assays were 42.7 and 10.66 µg/mL, respectively. In comparison with the standard, kava root extracts showed weaker antioxidant abilities. As shown in table 2, the antioxidant property of tested extracts decreased in the following order: EtOAc > water > chloroform > acetone and hexane. The greatest radical scavenging activity of EtOAc extract revealed that it contains the most abundant antioxidant compounds. Therefore, this fraction was chosen to further isolation and purification.

B. Isolation and Purification of Antioxidant Compounds by Column Chromatography

The isolated metabolites were characterized and identified by GC-MS, ESI/MS, and ^{1}H and ^{13}C NMR analyses. The structure elucidation of compounds C1, C2, C5, and C6 was described previously in Van et al., 2018.

The chemical structures of C3 and C4 were determined based on spectroscopic analyses and compared with previous literature data. The obtained compounds belong to the group of flavonoids: 5-hydroxy-4′,7-dimethoxyflavanone (C1), matteucinol (C2), 5,6-dihydroxy-7-methoxyflavone (C3) and kavalactones: 5,6-dehydrokawain (DK) and 5,6-dihydromethysticin (C4), DK (C5) and yangonin (C6) (Table 1, Figure 2).

C. Antioxidant Activity of Isolated Compounds

The antioxidant activity of the isolated constituents estimated by ABTS and DPPH methods is presented in Table 3. The results varied among tested compounds and studied assays.

The flavonoid compounds including C1, C2, C3 and a kavalactone mixture C4 were effective in scavenging ABTS free radicals. The ABTS scavenging activities of four fractions were followed the order: C3> C2> C4> C1. Whilst, the two kavalactone mixtures (C4 and C5) and the flavonoid C3 were able to reduce the stable DPPH radicals and their antioxidant capacities can be ranked as follows: C3> C4> C5. Among six fractions isolated from the EtOAc extract, C3 showed the highest inhibitory potential against ABTS and DPPH free radicals with similar IC_{50} results were 75.2 and 75.1 µg/mL, respectively. By contrast, the kavalactone C6 exhibited a negligible antioxidant activity.

However, the isolated compounds had lower inhibitory effects than positive control BHT on both antioxidant assays.

In the present study, the flavonoid C3 was the first time identified, purified in kava root Comparing with earlier reports concerning antioxidant activity of constituents isolated from kava root, this compound was observed significantly stronger antioxidant activity.

The result of this study highlighted the importance of the combination of hexane and ethyl acetate at 9:1 ratio in searching antioxidants from EtOAc extract of Piper methysticum root by column chromatography. This protocol may be utilized to isolate potential antioxidants from other plants belonging to Piperaceae family, which have been demonstrated to possess high antioxidant properties such as Piper cubeba and Piper nigrum (Nahak et al., 2011).

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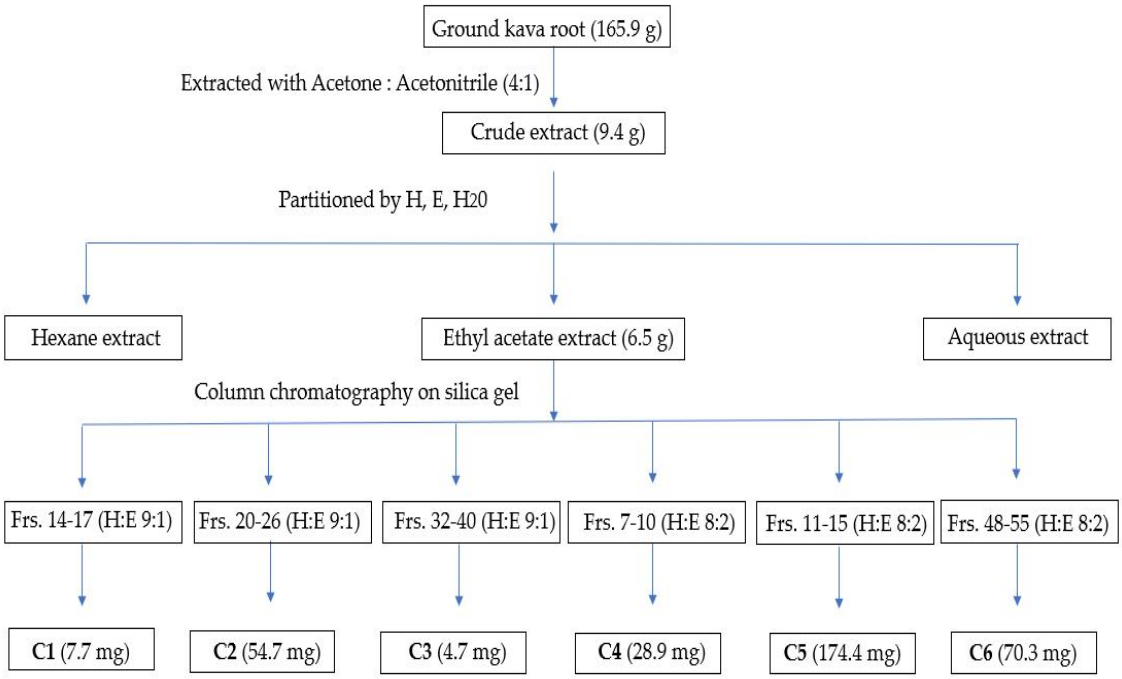
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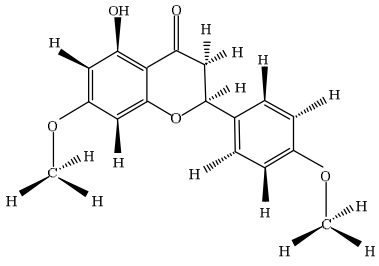


H: hexane, E: ethyl acetate, H20: water, Frs.: fractions

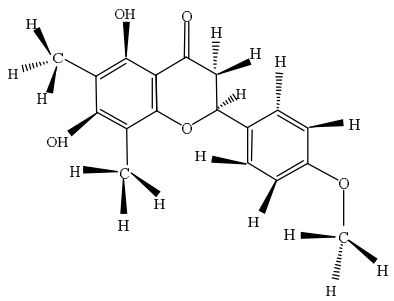
Figure 1. Fractionation and column chromatography separation of kava root

Table 1. Identification of isolated compounds from EtOAc extract of kava root by GC-MS, ESI-MS, ^{1}H and ^{13}C NMR.

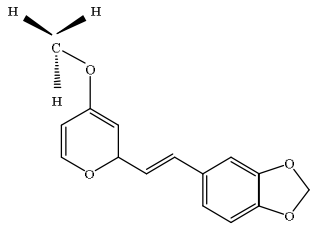
Code	Retention time	Peak area (%)	Compounds	Chemical formula	Molecular weight	Chemical class
C1	23.4	93.28	5-hydroxy-4',7-dimethoxyflavanone	C17H16O5	300	Flavanone
C2	25.37	90	5,7-(OH)2-4'-one-6,8-dimethylflavanone	C18H18O5	314	Flavanone
C3	21.67	97.17	5,6-dihydroxy-7-methoxyflavone	C16H12O5	284	Flavanone
C4	20.84	72.05	5,6-dehydrokawain	C14H12O3	228	Kava lactone
	22.26	27.95	5,6-dihydromethysticin	C15H12O5	272	Kava lactone
C5	20.8	94.66	5,6-dehydrokawain	C14H12O3	228	Kava lactone
C6	23.34	93.64	Yangonin	C15H14O4	258	Kava lactone



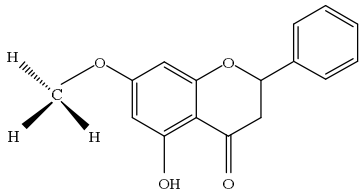
5-Hydroxy-4′,7-dimethoxyflavanone (C1)



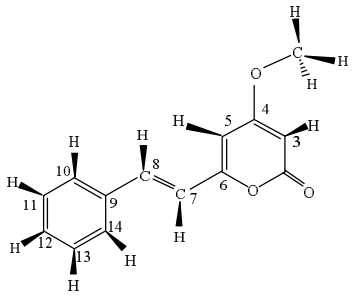
Matteucinol (C2)



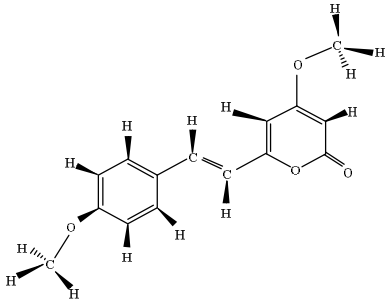
5,6-Dehydromethysticin (C4)



5,6-Dihydroxy-7-methoxyflavone (C3)



5,6-Dehydrokavain (DK) (C4, C5)



Yangonin (C6)

Figure 2. Chemical structure of bioactive constituents from P. methysticum root

Table 2. Antioxidant activity of Kava root extracts by ABTS and DPPH radical scavenging expressed as IC_{50} values

Extracts	IC_{50} (µg/mL)	
	ABTS	DPPH
Hexane	1705±334 ab	-
Chloroform	1300.1±65.4 bc	2397±80.1 c
Ethyl Acetate	1009±24.8 c	943±46.7 bc
Acetone	1943±254 a	3135±1494 a
Water	1012.1±44.8 c	972.9±31.4 bc
BHT	42.7±5.65 d	10.66±0.532 c

Data are presented as means ± SD (standard deviation). Mean values with different lowercase letters indicate significant differences in the same column ($p < 0.05$).

Table 3. Antioxidant activity of compounds isolated from kava root

Compounds	IC_{50} (µg/mL)	
	DPPH	ABTS
C1	-	203.5±5.5 a
C2	-	173.1±7.5 b
C3	75.1±2.9 c	75.2±8.4 c
C4	1210.2±94.2 b	181.7±8.8 b
C5	2238.6±377.0 a	-
C6	-	-
BHT	9.3±1.1 c	45.7±1.2 d

Data are presented as means ± SD (standard deviation). Mean values with different lowercase letters indicate significant differences in the same column ($p < 0.05$).

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