Flavonoid glycosides from leaves and straw of *Oryza sativa* and their effects of cytotoxicity on a macrophage cell line and allelopathic on weed germination

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**ABSTRACT**

Five new flavonoids namely, 5-hydroxy-6-isoprenyl-7,4'-dimethoxyflavonol-3-O-β-D-arabinofuranoside (1), 5,7-dihydroxy-4'-methoxyflavone-7-O-β-D-arabinopyranosyl-2'-O-decan-1'-oate (2), 3-butanoyl-5,6,8-trihydroxy-7,4'-dimethoxyflavonol-5-O-β-D-glucopyranoside (3), 7,4'-dimethoxy-5-hydroxyflavone-5-O-α-L-arabinopyranosyl-(2'→1')-O-α-D-arabinopyranoside (4), and 5,6-dihydroxy-7,4'-dimethoxyflavone-5-O-α-D-glucopyranoside (5), together with two known compounds, were isolated from the methanol extract of *Oryza sativa* leaves and straw. Their structures of new compounds were elucidated by 1D and 2D NMR spectral methods, viz: COSY, HMBC and HSQC aided by mass techniques and IR spectroscopy. The cytotoxicity of these compounds (1–7) were assessed by using (RAW 264.7) mouse macrophages cell line, and allelopathic effects of compounds (1–7) on the germination characteristics of barnyardgrass (*Echinochloa oryzicola*) and pigweed (*Chenopodium album*) were also evaluated. The compounds 1, 6 and 7 showed cytotoxicity and compounds 1–7 exhibited significant inhibitory activity on the seed germination of weed species.

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**1. Introduction**

Rice (*Oryza sativa*) is the principal cereal food in Asia and the major staple of the majority of the population. It generally occurs as two types, with white and colored hulls, although the white hulled variety is more common (85%). The germination of rice seed is of great agricultural importance, and it has long been known to be influenced by compounds present in the seed coat (hull) (Dutta, 1973).

Naturally occurring diterpenes, momilactones derived from rice have exhibited significant biological activities including as growth and germination inhibitors, herbcidical, algicidal, as well as potent inhibitory effects on several weeds and other activity (Kato et al., 1973; Kato et al., 1977; Kato-Naguchi et al., 2002; Kato-Naguchi and Ino, 2003). Earlier phytochemical investigation of rice husks, straw and leaves have led to the discovery of many classes of compounds and biological activities have been reported (Chung et al., 2005a,b; Chung et al., 2006a,b; Chung et al., 2007a,b; Ahmad et al., 2013; Chung et al., 2017).

This paper deals with the isolation and structure elucidation of five new flavonoid glycosides, (1–5) on the basis of \(^1\)H and \(^13\)C NMR spectroscopic studies, including 2D-NMR COSY, HSQC, HMBC and chemical reactions from *O. sativa*. This is the first report of isolation of flavonoid glycosides (1–5; Fig. 1) along with two known compounds (6–7, Fig. 2; Meyer et al., 2006). The cytotoxicity of the new and known compounds (1–7) were evaluated in a macrophage cell line RAW 264.7 by using an MTT assay and evaluated for their allelopathic effect on barnyardgrass (*Echinochloa oryzicola*) and pigweed (*Chenopodium album*), and characterization of weed seed...
germination and morphology was accomplished by treatment with different concentrations of the purified natural products are discussed. The objective of the present investigation was to report some of the new findings in the form of natural products and biological activities of compounds (1–7) from leaves and straw of O. sativa.

2. Experimental

2.1. General experimental procedures

Melting points of the compounds were determined using a model IA9100 melting point apparatus (Electrochemical Engineering, Seoul, South Korea). Optical rotations were measured on a model AA-10 polarimeter (Instrument Ltd., Seoul, Korea). Ultraviolet (UV) spectra were collected on a TU-1800PC UV–vis spectrophotometer (Instrument Ltd., Seoul, Korea). Infrared (IR) spectra were recorded on a Thermo Scientific FT-IR model Nicolet 6700 spectrophotometer (Waltham, MA, USA). Both nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance-600 spectrometer (Billerica Massachusetts (MA) using deuterated solvents. NMR spectra were recorded in deuterated chloroform, pyridine-d$_5$, and methanol-d$_4$, using tetramethylsilane (TMS) as an internal standard, with chemical shifts expressed in parts per million (δ) and coupling constants (J) in Hertz. High-resolution
Electrospray ionization Fourier transform (ESI/FT) mass spectra were recorded on a Thermo-Finnigan LTQ-Orbitrap instrument (Thermo Scientific, Bartlesville, OK, USA) equipped with a Dionex U 3000 HPLC system. All chemicals were of analytical grade. n-Hexane, ethyl acetate (EtOAc), methanol, ethanol, sulfuric acid (H2SO4), and vanillin were purchased from Daejung Chemicals (Seoul, South Korea). Normal thin layer chromatography (TLC) was performed on precoated silica gel 60 F254 plates (Merck). TLC plates were visualized using a 5% H2SO4 in ethanol spray reagent. Column chromatography (CC) was performed using silica gel (70–230 mesh from Merck) and LiChroprep RP-18 [40–63 μm; octadecyl silica (ODS) gel, Merck]. The authentic standards of chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material

The rice plant (O. sativa, leaves and straw) used in the present study was collected after the harvesting of rice cereal at Konkuk University Experimental Farm, Yeoju, South Korea, in September/October 2013. The collected samples were dried in the laboratory at a temperature range of 30–35°C for 3 weeks, with some modifications to the procedure performed in a previous study. A voucher specimen (reference code ILPUM variety) was dried and deposited in the herbarium of the Department of Applied Life Science, Konkuk University, Seoul, South Korea.

2.3. Extraction and isolation

Dried rice plant (2.4 kg, powdered leaves and straw) was immersed in methanol (10 L/3) for 1 week at room temperature (25–30°C). Then, the supernatant was concentrated under vacuum to yield 190 g of extract. This freeze-dried extract was again dissolved in methanol to remove fat and kept refrigerated for 3 h. The fat was crystallized and filtered through a sintered funnel. The filtrate was then concentrated to obtain 132 g of extract.

Table 1

| Position | 1 | 2 | 3 | 4 | 5 |
|----------|---|---|---|---|---|
| 1' | – | – | – | – | – |
| 2' | 7.42 d (8.0) | 7.01 d (8.5) | 7.04 d (8.0) | 7.36 d (9.0) | 7.01 d (8.5) |
| 3' | – | – | – | – | – |
| 4' | – | – | – | – | – |
| 5' | 7.16 d (8.5) | 6.98 d (9.0) | 6.74 d (8.5) | 6.78 d (9.0) | 6.75 d (8.5) |
| 6' | 7.37 d (8.5) | 6.98 d (9.0) | 6.90 d (8.5) | 7.34 d (9.0) | 7.17 d (8.5) |
| 1'' | 2.93 d (7.0), 2.90 d (7.5) | 5.01 d (7.0) | – | 5.40 d (4.5) | 5.01 d (4.5) |
| 2'' | 6.65 d (7.5) | 4.21 m | 2.59 t (8.5) | 4.63 m | 3.80 m |
| 3'' | – | 3.87 m | 1.22 m | 4.28 m | 3.71 m |
| 4'' | 2.05 br s | 3.82 m | 0.95 t (6.3) | 3.64 m | 3.67 m |
| 5'' | 2.01 br s | 3.78 d (4.5) | – | 3.29 m | 4.20 m |
| 6'' | – | 3.76 d (5.0) | – | – | 3.29 br s |
| 1''' | 6.40 d (7.5) | – | 4.80 d (7.0) | 5.13 d (4.5) | – |
| 2''' | 3.88 m | 2.24 t (7.0) | 3.77 m | 4.36 m | – |
| 3''' | 3.85 m | 2.08 m | 3.57 m | 4.13 m | – |
| 4''' | 4.56 m | 1.55 m | 3.46 m | 3.47 m | – |
| 5''' | 3.56 d (7.0), 3.54 d (7.0) | 1.30 m | 3.85 m | 3.17 m | – |
| 6''' | – | 1.27 br s | 3.50 d (7.0) | – | 3.48 (7.0) |
| 7''' | – | 1.27 br s | – | – | – |
| 8''' | – | 1.27 br s | – | – | – |
| 9''' | – | 1.27 br s | – | – | – |
| 10''' | 0.94 t (7.5) | – | – | – | – |
| (OMe) | 3.30 s | – | 3.82 s | 3.87 s | 3.85 s |
| (OMe) | 3.29 s | – | 3.75 s | 3.86 s | 3.77 s |

*a Coupling constants in parenthesis are given in hertz.
The methanol extract (132 g) was subjected to normal-phase CC on silica gel (70–230 mesh, 1.2 kg, 1500 ± 45 mm), yielding 50 fractions (each fraction 500 mL) with the following eluents: fraction 1–5 in hexane, fractions 6–10 in hexane:EtOAc (H:Et; 8:2), fractions 11–15 in H:Et (6:4), fractions 16–20 in H:Et (4:6), fractions 21–25 in H:Et (8:2), fractions 26–30 in EtOAc, fractions 31–35 in EtOAc:MeOH:Et:Me (Et:Me; 9:5:0.5), and fractions 36–40 in Et:Me (Et:Me; 8.5:1.5), and fractions 45–50 in Et:Me (8:2). Fractions 6–9 were crystallized and yielded β-sitosterol (23 mg) after purification by CC. Compound 6 (54 mg) was obtained (34 mg) from fraction 10 after CC in a small column with methanol:water (80, 60, 40, 20, 10, and 0% water) to yield three new compounds: 1 (52 mg), 2 (49 mg), and 3 (39 mg). Fractions 36–40 were combined after chromatography on a silica gel column with chloroform and methanol, recrystallized over LiChroprep RP-18, and eluted sequentially with methanol containing 80, 60, 40, 20, 10, and 0% water to yield four new compounds: 1 (39 mg), 2 (49 mg), 3 (54 mg), and β-sitosterol-β-β-glucoside (29 mg).

### Table 2

| Position | 1     | 2     | 3     | 4     | 5     |
|----------|-------|-------|-------|-------|-------|
| 1        | 147.9 | 164.8 | 147.0 | 164.7 | 164.0 |
| 3        | 133.1 | 104.9 | 136.7 | 134.3 | 104.9 |
| 4        | 179.2 | 183.5 | 175.1 | 183.3 | 180.2 |
| 5        | 160.9 | 163.0 | 150.4 | 163.0 | 163.1 |
| 6        | 118.6 | 100.2 | 132.6 | 102.3 | 149.6 |
| 7        | 160.4 | 165.9 | 162.8 | 165.4 | 164.8 |
| 8        | 90.0  | 95.2  | 131.3 | 96.0  | 96.3  |
| 9        | 151.1 | 159.1 | 148.9 | 158.4 | 160.1 |
| 10       | 103.5 | 105.7 | 104.9 | 101.7 | 107.2 |
| 11       | 124.2 | 127.5 | 129.4 | 121.0 | 127.8 |
| 12       | 130.9 | 136.7 | 128.9 | 127.9 | 129.5 |
| 13       | 116.5 | 111.8 | 115.2 | 116.5 | 115.6 |
| 14       | 145.9 | 144.6 | 146.2 | 144.4 | 144.8 |
| 15       | 112.5 | 115.8 | 115.3 | 112.3 | 114.6 |
| 16       | 128.3 | 128.7 | 128.8 | 128.9 | 128.8 |
| 17       | 41.4  | 105.4 | 172.1 | 103.2 | 105.2 |
| 18       | 123.7 | 87.5  | 38.6  | 89.6  | 77.3  |
| 19       | 141.6 | 74.5  | 28.2  | 78.6  | 74.4  |
| 20       | 26.4  | 74.0  | 18.2  | 74.3  | 71.2  |
| 21       | 5.6   | 62.8  | 182.7 | 56.7  | 78.6  |
| 22       | -     | -     | -     | -     | 62.6  |
| 23       | 116.6 | 178.2 | 103.1 | 101.3 | -     |
| 24       | 74.0  | 38.2  | 74.9  | 79.7  | -     |
| 25       | 72.3  | 35.5  | 71.3  | 75.2  | -     |
| 26       | 19.0  | 30.4  | 68.6  | 71.6  | -     |
| 27       | 69.8  | 30.3  | 78.1  | 62.4  | -     |
| 28       | -     | 30.1  | 62.5  | -     | -     |
| 29       | -     | 26.1  | -     | -     | -     |
| 30       | -     | 22.6  | -     | -     | -     |
| 31       | 10.0  | -     | -     | -     | -     |

The IR spectra of a previously isolated compound. After mixing fractions 1–5 are in pure form and its identity was confirmed by comparison with an authentic sample from Sigma and with the spectra of a previously isolated compound. After mixing fractions 1–5 are in pure form and its identity was confirmed by comparison with an authentic sample from Sigma and with the spectra of a previously isolated compound.
2.4.3. Weed collection and dormancy breakage

The seeds of two prominent weeds (Echinochloa oryzicola and Chenopodium album L.) found in the rice paddies of Korea were collected in 1997 and 1998. The seeds were dried (12.04% water content based on fresh weight) and stored at 5 °C in the dark. Before the start of the experiment, the seeds were scarified with H2SO4 (98%) for 30 min to render the seed coat permeable to water. Pigweed seeds were rinsed several times with distilled water to remove traces of H2SO4. The seeds of barnyardgrass were soaked in distilled water for one day to remove the germination inhibiting compound in the seed coat (water priming, WP). Their germination was determined and was >80% in all cases.

2.4.4. Seed germination inhibition assay

A bioassay based on seed germination was used to assess the inhibitory activity of the isolated compounds on seed germination. Test solutions at concentrations of 0, 100, 500, and 1000 μM were prepared by serial dilution of stock solutions of the constituents (1 M in DMSO). Test solutions of pyributicarb (PBC), an inhibitor of germination, were also prepared by serial dilution in the same solvent. Prior to the seeds being sown in petri dishes (9 cm in diameter), the barnyardgrass and lamb’s quarters seeds were surface sterilized with distilled water, then with 95% ethanol for 15 s, then rinsed with distilled water. Ten seeds were sown in each petri dish. Each test solution (7 mL) was poured on double-layered filter paper placed in the petri dishes. Distilled water and a DMSO solution were taken as the controls. All treatments were replicated three times. The germination tests were performed in a germinator with 70% relative humidity at 25 °C under 16-h light and 8-h dark lighting conditions. To measure the germination indices (GIs), the germinated seeds were counted daily. At the end of the last day of germination, the seedling growth and indices, including the final germination percentage (FGP), GI, the coefficient of velocity of germination (CVG), germination speed (GS), and the mean germination time (MGT), were calculated using the following formulas:

\[ (1) \text{FGP:} \]

\[ \text{FGP} = \frac{\text{Ng}}{\text{Ng} \times \text{Nt}} \times 100 \]

\[ \text{Ng} = \text{Total number of seeds germinated} \]

\[ \text{Nt} = \text{Total number of seeds evaluated} \]

\[ (2) \text{GI:} \]

\[ \text{GI} = \left(13 \times N_1\right) + \left(12 \times N_2\right) + \ldots + \left(1 \times N_{13}\right) \]

\[ N_1, N_2 \ldots \text{are the number of germinated seeds on the first day, second, and other days, and the numbers 9, 10 and ... are the weights imposed on the number of seeds germinated at first day, second, and other days, respectively.} \]

\[ (3) \text{CVG:} \]

\[ \text{CVG} = 100 \times \frac{\sum \text{Ni}}{\sum \text{NiTi}} \]

\[ \text{Ni} = \text{Number of germinated seeds per day} \]

\[ \text{Ti} = \text{Days from the start of the experiment} \]

\[ (4) \text{GS was calculated according to the method of Magour:} \]

\[ \text{Gs GS} = \sum \text{Si/Di} \]

\[ \text{Si} = \text{Number of seeds germinated on the i^{th} day} \]
Di = Number of days to counting n\textsuperscript{th}

(5) MGT:

\[ \text{MGT} = \frac{\sum \text{NiTi}}{\sum \text{Ni}} = \frac{100}{\text{CVG}} \]

Ni = Number of seeds germinated per day
Ti = Days from the beginning of the experiment

3. Results and discussion

The methanol extract of leaves and straw of Oryza sativa was column chromatographed and obtained seven compounds (1–7). Their structures were elucidated on the basis of spectroscopic data.

Compound 1 was isolated as a yellow semi-solid, [\(\alpha\)]\text{D}\text{21} = 37.2 (c 0.1, MeOH). The molecular formula C\text{27}H\text{31}O\text{10} was established by \text{13}C NMR and HRESI/FTMS data (m/z 515.1898 [M + H]\text{+} calcd

Fig. 3. HMBC correlation of compounds 1–5.
for 515.1917), suggesting 13 indices of hydrogen deficiency. The UV absorption maxima at 270, 310 and 328 nm were characteristic of a flavonoid (Harborne and Williams, 1975; Markham, 1982; Chung et al., 2009; Mabry et al., 1970). The IR spectrum displayed characteristic absorption bands for hydroxyl groups (3415 and 3369 cm⁻¹), carbonyl groups (1662 cm⁻¹) and aromatic rings (1614, 1590 cm⁻¹). The mass fragmentation patterns of compound 1 are shown in Fig. 1.

The ¹H NMR spectrum of 1 indicated a flavonoid moiety, as it displayed four one-proton meta-coupled doublets at δH 7.42 (J = 8.0 Hz), 6.77 (J = 8.0 Hz), 7.16 (J = 8.5 Hz), and 7.37 (J = 8.5 Hz) assigned to the H-2', H-3', H-5' and H-6' protons. Two one-proton doublets at δH 5.56 (J = 7.0 Hz) and 3.54 (J = 7.0 Hz) assigned to the H-2αα and H-2ββ' proton signals, respectively. Two three-proton broad singlets at δH 3.29 and 3.30 and another two broad singlets at δH 2.01 and 2.05 were assigned to the methylene protons on C-7 and C-4α in flavone moiety and Me-4α and Me-5α protons, respectively. The methylene and methine protons in isoprenyl moiety attached to flavone moiety appeared at δH 2.90 (J = 7.5 Hz), 2.93 (J = 7.0 Hz), and 6.65 (J = 7.5 Hz) and were assigned to the H-3′-a, H-3′-b, and H-2′ protons. The methine protons in the sugar that appeared as one doublet δH 6.40 (J = 4.0 Hz) and three multiplets δH 3.88, 3.85, and 4.56 were assigned to the H-1′ and H-2′ and H-3′, H-4′ protons. The sugar unit in 1 was identified as β-arabinofuranoside by analyzing the coupling constant of the anomeric proton signal, which is evident as a one-proton doublet at δH 5.01 (J = 7.0 Hz). The methine protons H-2', H-3', H-4' and H-6' in sugar assigned as multiplets at δH 4.21–3.82, and methylene proton in sugar H-2α and H-2β' as doublets at δH 3.78 (J = 4.5 Hz) and 3.76 (J = 5.0 Hz). A three-proton triplet at δH 0.94 (J = 7.5 Hz) was attributed to the C-10' primary methyl protons. Methylenic protons resonated between δH 2.24 and 1.27, and a three-proton broad signal at δH 3.32 was assigned to the C-4' methoxy protons. The ¹³C NMR spectrum of 2 showed signals for the C-4 flavone carbonyl carbon at δC 183.5, for the other flavone carbons between δC 165.9 and 95.2, for the anomic carbon at δC 105.7 (C-1′), and ester carbon at δC 178.2 (C-1′′), for an aliphatic chain methyl carbon at δC 14.1 (C-8′′), for a methoxy carbon at δC 56.6, and for the other sugar carbons resonated between δC 87.5 and 62.8. The ¹H NMR signals in the deshielded region at δH 5.42 (H-2) as well as the corresponding carbon signals at δC 87.5 (C-2′′) suggested a (2′→1) glycosidic linkage and confirmed the attachment of the ester group at C-2′. The appearance of the C-7 signal at δC 165.9 in the ¹³C NMR spectrum was compared with literature data (Chung et al., 2009). The ¹H–¹H COSY spectrum of 2 showed correlations of H-6 with H-8; H-2′ with H-3′ and H-6′; H-2′ with H-1′ and H-3′. The HMBC spectrum of 2 exhibited correlations of H-4′ with C-4′, C-5′; H-1′ with C-7′; H-6′ with C-5′, C-8′; H-8′ with C-6′, C-10′; H-3′, H-5′ with C-1′; CH3O with C-4′ (Fig. 3). The HSQC correlations were used to assign all protons and carbons atoms in the molecule and some important correlations are H-1′ with C-1′ and C-4′ interacted with H-5′. The ¹H and ¹³C NMR signals of the flavone moieties were compared with those described for similar compounds in the literature (Agrawal, 1989; Waffo et al., 2006). The ¹H–¹H COSY spectrum of 1 showed correlations of H-2′ with H-3′ and H-6′; H-5′ with H-6′; H-1′ with H-2′ and H-1′ with H-2′′. The appearance of the C-3′ signal at δC 133.1 in the ¹³C NMR spectrum supported the attachment of the sugar moiety at this carbon (Mariani et al., 2008; Schliemann et al., 2006; Saleem et al., 2006). The HMBC spectrum of 1 exhibited correlations of H-1′′ with C-3; H-5 with C-5′, C-6; H-8 with C-6′, C-10; CH3O with C-7, H-3′, H-5′ with C-1′ and CH3O with C-4′ (Fig. 3). The HSQC correlations were used to assign all protons and carbons atoms in the molecule; some common correlations are H-1′′ with C-1′. Acid hydrolysis of 1 yielded sugar part (see Section 2). According to the analysis of the spectroscopic data given above and the 2D NMR data (COSY, HSQC and HMBC) as well as the results from the chemical reaction, the structure of 2 has been established as 5,7-dihydroxy-4-methoxyflavone-7-O-β-D-arabinopyranosyl-2′-n-decan-1-oate, which is a new compound was as shown in Fig. 1.

Compound 3 was isolated as a yellow solid, [α]D 31.0 (c 0.1, MeOH). The molecular formula C21H19O14 was established by ¹³C NMR and HR-ESI/FTMS data (m/z 579.1661 [M + H]+ calc for 579.1669), suggesting 13 indices of hydrogen deficiency. The UV absorption maxima at 277, 312, and 339 nm were characteristic of a flavonoid (Harborne and Williams, 1975; Markham, 1982; Chung et al., 2009; Mabry et al., 1970). The IR spectrum displayed characteristic absorption bands for hydroxyl groups (3515, 3450 and 3361 cm⁻¹), ester functionalities (1722 cm⁻¹), carbonyl groups (1680 and 1655 cm⁻¹). The mass fragmentation patterns of compound 3 are shown in Fig. 1.

The ¹H NMR spectrum of 3 indicated a flavonoid moiety, as it displayed four ortho-coupled double doublets at δH 7.04 (J = 8.0 Hz), 6.70 (J = 8.0 Hz), 6.74 (J = 8.5 Hz) and δH 6.90 (J = 8.5 Hz) assigned to H-2′, H-3′, H-5′ and H-6′ protons, suggesting a 4'-oxygenated substitution pattern in ring B and ortho-coupled protons characteristic of an AA′XX′ spin system of a para-substituted phenyl ring in ring B. The sugar unit in 3 was identified as β-arabinopyranoside by analyzing the coupling constant of the anomeric proton signal, which is evident as a one-proton doublet at δH 5.74 (J = 7.0 Hz). The remaining H-2′, H-3′, and H-4′ sugar protons appeared as multiplets at δH 3.86–3.57. A three-proton triplet at δH 3.65 (J = 6.3 Hz) was attributed to C-4’ primary methyl protons, methylene protons resonated between δH 2.59–1.22, and a three-proton two
The 13C NMR spectrum of 3 showed signals for the C-4 flavone carbonyl carbon at δC 175.1, for the other flavone carbons resonated between δC 162.8 and 104.9, for the anomeric carbon at δC 103.1 (C-1'), for the ester carbon at δC 172.1 (C-3'), for an aliphatic chain methyl carbon at δC 18.2 (C-4'), for a methoxy carbons at δC 56.4, 56.8, and for the other sugar carbons resonated between δC 78.1 and 62.5. The 1H and 13C NMR shifts of the flavone moieties were compared with those described for similar compounds in the literature (Agrawal, 1989). The 1H–1H COSY spectrum of 3 showed correlations of H-2’ with H-3’ and H-6’; H-2” with H-3” and H-1” with H-2”. The glucopyranosyl residue was located at the C-5 position of flavones skeleton according to long-range HMBC correlations between C-5 at δC 150.4 and the anomeric H-1” at δH 4.80 was compared with literature data (Zahir et al., 1999). The HMBC spectrum of 3 exhibited correlations of CH2O with C-7; H-6 with C-5, C-6; H-1” with C-5, C-5”; H-2” with C-4”; H-3’, 5’ with C-1 and H2CO with C-4’ (Fig. 3). The HSQC correlations were used to assign all protons and carbons atoms in the molecule and some important correlations are H-1” with C-1” and H-4” interacted with C-4’”. Acid hydrolysis of 3 yielded sugar part (see Section 2). According to the analysis of the spectroscopic data given above and the 2D NMR data (COSY, HSQC, and HMBC) as well as the results from the chemical reaction tests, the structure of 3 has been established as 3-butanoyl-5,6,8-trihydroxy-7,4’-dimethoxyflavonol-5-O-α-D-glucopyranoside, which is a new compound.

Compound 4 was isolated as a yellow solid, [α]D21 = 29.2 (c 0.1, MeOH). The molecular formula C27H31O13 was established by 13C NMR and HR-ESI/FTMS data (m/z 563.1711 [M + H]+ calc for 563.1720), suggesting 13 indices of hydrogen deficiency. The UV absorption maxima at 269, 309, and 329 nm were characteristic of a flavonoid (Harborne and Williams, 1975; Markham, 1982; Chung et al., 2009; Mabry et al., 1970). The IR spectrum displayed absorption maxima at 1663 cm−1 (C=O, carbonyl groups), 1647, 1590, and 1504 cm−1 (phenyl rings).

The 1H NMR spectrum of 4 indicated a flavone moiety, as it displayed two one-proton meta-coupled singlets at δH 6.46 and 6.68 and ortho-coupled doublets at δH 7.36 (J = 9.0 Hz), 6.70 (J = 9.0 Hz), 6.78 (J = 9.0 Hz) and 7.34 (J = 9.0 Hz) assigned to H-2’, H-3’, H-5’, and H-6’ protons, suggesting a 4’-oxygenated substitution pattern in ring B, a meta-coupled AX system corresponding to the H-6 and H-8 protons in ring A, and ortho-coupled protons characteristic of an AA’XX’ spin system of a para-substituted phenyl ring in ring B. The sugar unit in 4 was identified as α-arabinopyranosyl by analyzing the coupling constant of the anomeric proton signals, which are evidence as two one-proton doublets at δH 5.40 (J = 4.5 Hz) and 5.13 (J = 4.5 Hz). The sugar protons H-2’, H-2”; H-3’, H-3”; and H-4”, H-4” appeared as multiplets between at δH 4.63 – 3.47, and the methylene sugar protons H2-5” and H-2-5” appeared as multiplets at δH 3.29 and 3.17.

The 13C NMR spectrum of 4 showed signals for the C-4 flavone carbonyl carbon at δC 183.3, for the other flavone carbons between δC 164.7 and 96.0, for the anomeric carbons at δC 105.3 (C-1”) and 101.3 (C-1”), for methoxy carbons at δC 57.0 and 56.3, and for the other sugar carbons between δC 89.6 and 62.43. The 1H NMR signals in the deshielded region at δH 4.63 (H-2”) as well as the corresponding carbon signals at δC 89.6 suggested a (2 → 1) glycosidic linkage. The 1H and 13C NMR values of the flavone moiety were compared with those described for similar compounds in the literature (Agrawal, 1989). The arabinopyranosyl residue was located at the C-5 position of flavones skeleton according to long-range HMBC correlations between C-5 at δC 163.0 and the anomeric H-1” at δH 5.40 as well as H-6 at δH 6.46; these results were compared to the literature data (Zahir et al., 1999).

The 1H–1H COSY spectrum of 4 showed correlations of H-6 with H-8 and OMe (ring A); H-3’ with OMe (ring B); H-2’ with H-3’; H-5’ with H-6’; H-2” with H-1’. The HMBC spectrum of 4 exhibited correlations of CH2O with C-7; H-6 with C-5, C-7 and C-8; H-3 with C-2; H-3’, 5’ with C-1; CH2O with C-4’; H-1” with C-5 and H-1’” with C-2” (Fig. 3). The HSQC correlations were used to assign all protons and carbons atoms in the molecule and some important correlations are H-1” with C-1” and H-4” interacted with C-4’”. Acid hydrolysis of 4 yielded sugar part (see Section 2). According to the analysis of the spectroscopic data given above and the 2D-NMR data (COSY, HSQC and HMBC) as well as the results from the chemical reaction tests, the structure of 4 has been established as 7,4’-dime-thoxy-5-hydroxyflavone-5-O-α-D-arabinopyranosyl(2’→1”)-O-α-D-arabinopyranoside, which is a new compound (Fig. 1).

Compound 5 was isolated as a yellow solid, [α]D21 = 39.2 (c 0.1, MeOH). The molecular formula C23H25O11 was established by 13C NMR and HR-ESI/FTMS data (m/z 563.1711 [M + H]+ calc for 563.1720), suggesting 13 indices of hydrogen deficiency. The UV absorption maxima at 279, 313, and 334 nm were characteristic of a flavonoid (Harborne and Williams, 1975; Markham, 1982; Chung et al., 2009; Mabry et al., 1970). The IR spectrum displayed absorption maxima at 269, 309, and 329 nm were characteristic of a flavonoid.

Fig. 4. Percentage viability of RAW 264.7 cells exposed to the isolated chemical constituents from Oryza sativa (straw and leaves). DMEM (Dimethyl sulfoxide). Data are presented for 24 h and calculated from the absorbance values obtained from the MTT assay. The cell viability percentage was the mean absorbance of the seven allelopathic chemical constituents at different concentrations (100, 500, 1000 μM) divided by that of the corresponding control group. The bars represent the mean ± SD obtained from three independent experiments.
Chung et al., 2009; Mabry et al., 1970). The IR spectrum displayed characteristic absorption bands for hydroxy groups (3415, 3383, 3281 cm\(^{-1}\)), carbonyl groups (1701 cm\(^{-1}\)), and methine protons H-2\(=\) 8.5 Hz) assigned to the H-3, H-8, H-2\(\alpha\), and H-5 with OMe (ring B). The HSQC correlations were seen for the anomic proton signal, which is evident as a one-proton doublet at \(\delta_p\) 5.01 (J = 4.5 Hz). The other methine protons H-2\(\alpha\), H-3\, H-4\(\beta\), and H-5\(\alpha\) appeared as multiplets between at \(\delta_p\) 4.20–3.67, and methylene protons H-6\(\alpha\) appeared as a broad singlet at \(\delta_p\) 3.29. The \(^{13}\)C NMR spectrum of 5 showed signals for the C-4 flavone carbonyl carbon at \(\delta_C\) 180.2; for the other flavone carbons at \(\delta_C\) 164.0 (C-2), 104.9 (C-3), 163.1 (C-5), 149.6 (C-6), 164.8 (C-7), 96.3 (C-8), 160.1 (C-9), 107.2 (C-10), 127.8 (C-11), 129.50 (C-20), 115.69 (C-3), 154.8 (C-4), 114.64 (C-5), and 120.8 (C-1'); for the aromatic carbon at \(\delta_C\) 105.2 (C-1'); for methoxy carbons at \(\delta_C\) 57.0 and 56.4, and for the other sugar carbons between \(\delta_C\) 89.6 and 62.4. The \(^{1}\)H and \(^{13}\)C NMR values of the flavone moieties were compared with those described for similar compounds in the literature (Agrawal, 1989). Acid hydrolysis of 5 yielded flavones and sugar. The glucosyl residue was located at the C-5 position of flavones skeleton according to long-range HMBC correlations between C-5 at \(\delta_C\) 163.1 and the anomic H-1\(\alpha\) at \(\delta_H\) 3.67 was compared with literature data (Zahir et al., 1999).

The \(^{1}\)H–\(^{13}\)C COSY spectrum of 5 showed correlations of H-2\(\alpha\) with H-5\(\beta\), H-5\(\beta\) with H-6\(\alpha\); H-2\(\alpha\) with H-1\(\beta\), H-1\(\beta\) with OMe (ring B), H-3 with OMe (ring B). The HMBC spectrum of 5 exhibited interactions of H-3 with C-2, C-3; H-6 with C-1, C-2, C-5, and C-6; CO with C-7, C-8; H-2\(\alpha\) with C-3' (Fig. 3). The HSQC correlations were used to assign all protons and carbons atoms in the molecule and some important correlations are H-1’ with C-1’. Acid hydrolysis of 5 yielded sugar part (see Section 2). According to the analysis of spectroscopic data given above and the 2D NMR data (COSY, HSQC and HMBC) as well as the results from the chemical reaction tests, the structure of 5 has been established as 5, 6-dihydroxy-7, 4′-dimethoxyflavone-5-O-α-D-glucopyranoside, which is a new compound (Fig. 1).

\[4′\text{-methoxyapigenin (6),} \quad 5,7\text{-dihydroxy-4′-methoxyflavone-7-O-β-D-arabinopyranoside (7),} \quad \beta\text{-sitosterol,} \quad \beta\text{-sitosterol-β-D-glucoside were identified by comparison with literature data (Meyer et al., 2006). This is the first report of the isolation of compounds 6-7 from this plant.} \]

The cytotoxicity of the constituents (1–7) on a RAW 264.7 macrophage cell line were determined by the MTT assay, and the results are shown in Fig. 4. The data indicate that the cell viability rate decreased significantly (p < .05) compared to those of the control group as the concentrations of compounds 1, 6 and 7 increased. However, the cytotoxicity increased dose dependently.
In each experiment in the current study, the absorbances among the three parallel experiments were similar. Overall, the results showed that the inhibitory effect of compounds 1, 6, and 7 at 100 μM were very nearly similar, with only slight differences. According to the results, 5% DMSO in distilled water displayed a higher cytotoxic effect (79.9%) than DMEM (100%). When the concentration of compounds 2, 3, 4, and 5 in DMSO were 100 μM, the corresponding cell viability rate observed in RAW 264.7 cells was 101.1, 70.7, 69.9, and 75.8%, respectively. Compounds 3, 4, and 5 at 1000 μM had little or no toxicity, whereas compound 2 at higher concentrations inhibited cell growth. In addition, the viability rate of all chemical constituents did not exceed 69.4% at doses up to 1000 μM in Fig. 4.

The allelopathic effects of compounds 1–7 at different concentrations (100, 500, 1000 μM) on the tested germination parameters final germination percentage (FGP), mean germination time (MGT), germination speed (GS), coefficient of velocity of germination (CVG) and germination index (GI)] are shown in Table 3. The FGP, MGT, GS, CVG, and GI values with distilled water were 76.67%, 2.86 days, 7.67 days, 51.1 day⁻¹, and 1.53, respectively. The corresponding values with DMSO were 43.21%, 3.33 days, 4.33 days, 32.04 day⁻¹, and 0.87.

Applying pyributicarb (PBC) had negligible inhibitory effects on pigweed germination (Fig. 6). FGP was slightly affected by compounds 2, 5, 6, and 7; however, the GS and CVG values showed greater variability than the other germination parameters. The GS and CVG ratios decreased as the concentration of the chemical constituents increased. According to this result, in the presence of PBC, compounds 2, 5, 6, and 7 delayed the emergence of seeds. Germination is an important stage in plant growth, and these
The activity of enzymes such as protease, lipase, and α-amylase, which play an important role during germination, is inhibited under the influence of allelopathic chemicals. These compounds reduced and delayed germination, whereas, to the best of our knowledge, compounds 1, 3, and 4 drastically affected all germination parameters. Compound 3 inhibited FGP significantly more than the other chemical constituents. Compared to PBC and the other chemical constituents, compound 3 at 100 μM had the strongest allelopathic effect on pigweed seed germination and growth (Fig. 5).

Barnyardgrass germination was significantly inhibited at low PBC concentrations (100 μM) of PBC and was completely inhibited at moderate (500 μM) and high (1000 μM) PBC concentrations (Table 4). In general, all the isolated chemical constituents from rice inhibited the FGP of barnyardgrass with the range of 3.33–20.25% compared to 48.25% FGP for DMSO and 100% for distilled water. Compared to an MGT of 4.68, a GS of 4.33, a CVG of 21.67 and a GI of 23.09 for distilled water. For the isolated chemical constituents from rice, the MGT ratios ranged from 1 to 5.78, GS ranged from 1.87 to 0.60, CVG ranged from 8.33 to 1.43, and GI ranged from 7.87 to 3.56; by comparison, for distilled water, the MGT was 4.68, the GS was 4.33, the CVG was 21.67 and the GI was 23.09. The MGT value shows negligible changes compared to the other germination parameters. Conversely, the GS, CVG, and GI values differed from the aforementioned germination parameters. These parameters decreased as the concentrations of the isolated chemical constituents from rice decreased (Table 2), indicating that these chemical constituents decreased the intensity of germination induced by WP treatments and inhibited seed germination and seedling emergence. Many researchers have also reported that in weak or stored seeds, priming increased germination and emergence (Parrish and Leopold, 1978).

The seedling growth (hypocotyl and radicle) of barnyard grass was very sensitive to the inhibition of hypocotyl elongation (Fig. 6). Seed germination was inhibited completely at high concentrations (1000 μM) of compounds 3 and 4. Furthermore, all applied concentrations of compound 5 completely inhibited the germination and growth of barnyard grass.

Compounds 6 and 7 were originally isolated from rice leaf and straw as phytoalexins to participate in the defense of rice against pathogens, whereas, to the best of our knowledge, compounds 1, 3, 4 and 5 inhibited seed germination and growth of barnyard grass. The activity of enzymes such as protease, lipase, and α-amylase, which play an important role during germination, is inhibited under the influence of allelopathic chemicals. These compounds reduced and delayed germination, whereas, to the best of our knowledge, compounds 1, 3, and 4 drastically affected all germination parameters.
2, 3, 4, and 5 have not previously been reported in rice plants. Because they are substituted methoxy analogues of flavone, compounds 3 (5,6,8-trihydroxy-7,4'-dimethoxyflavanol-3-butanoyl-5-O-β-D-glucopyranoside) and 5 (5,6-dihydroxy-7,4'-dimethoxyflavone-5-O-α-D-glucopyranoside) could significantly inhibit the growth of pigweed and barnyardgrass. Biological trials were performed by method (Kong et al., 2004) showed the inhibitory activity of 5,7,4'-trihydroxy-3,5,7,4'-dimethoxyflavone against weeds of the species E. crusgalli, Cyperus difformis, and Cyperus iria.

Plant-plant interactions can be positive or negative and may depend on flavonoid concentrations (Chou, 1999). The negative interactions mainly involve inhibiting the germination of other plants' seedlings (Treutter, 2005). However, the precise mechanism by which flavonoids participate in allelopathy remains unknown. The potential ways in which these compounds can influence allelopathy may include inhibiting cell growth, disturbing ATP production, and hindering the proper functioning of auxins (Berhow and Vaughn, 1999). Flavanols were reported to provoke an abundance of reactive oxygen species, which activate the Ca²⁺ signal cascade and root system death (Bais et al., 2003).

These results suggested that the five new constituents and two known compounds isolated from rice might be used as natural herbicides in paddy fields. However, more experiments on the application rates or time of these compounds are needed to increase the efficacy of the isolated chemical constituents from rice. In this study, these isolated compounds were established as promising materials for the biological control of weeds such as pigweed and barnyardgrass. This method is safer for human health and the environment than herbicide application. However, further study is needed to verify the mechanism underlying the allelopathic characteristics of flavonoid and its substituted analogues. In addition, the extent to which dependence on synthetic herbicides can be reduced by this approach should be investigated.

4. Conclusions

To the best of our knowledge, there has been no prior report on the phytochemistry of straw and leaves in this paper isolation five new and two known compounds along with its biological activities including cytotoxicity and allelopathic activities on weed germination.

Notes

The authors declare that they have no conflicts of interest.

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