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ORIGINAL PAPER

In vivo anthelmintic effect of flavonol rhamnosides from *Dryopteris crassirhizoma* against *Dactylogyrus intermedius* in goldfish (*Carassius auratus*)

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Abstract The purpose of the present study was to assess the anthelmintic property of plant-derived polyphenolic compounds extracted and isolated from *Dryopteris crassirhizoma* against *Dactylogyrus intermedius* in goldfish. The active ethyl acetate extract was loaded on an open silica gel column and eluted with chloroform–methanol. According to ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and mass spectral data, the structures of three purified compounds were identified as protocatechuic acid, sutchuenoside A, and kaempferitrin. Among these compounds, sutchuenoside A and kaempferitrin were observed to be effective with median effective concentration (EC_{50}) of 3.01 and 2.71 mg L^{-1}, respectively. The alterations in the tegument of the parasites treated with isolated compound were examined using scanning electron microscopes. Ultrastructural micrographs revealed shrinkage of body surface, dense tegumental folds, and disheveled protuberances. The structural deformities in the treated parasites were indicative of an efficient anthelmintic activity of the isolated compound kaempferitrin. In addition, the 48-h median lethal concentration for sutchuenoside A and kaempferitrin against goldfish were 12.03- and 11.98-fold higher than corresponding EC_{50}. The present results showed that ethyl acetate extract of *D. crassirhizoma* may be considered as a potent source, and sutchuenoside A and kaempferitrin as new natural parasitic agents against *D. intermedius*.

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Introduction

Aquaculture in Asia-Pacific region, particularly in China, the largest producer with its output, is of major importance in the global context (FAO 2010). However, bacteria, virus, and parasite are reportedly associated with a series of infectious disease outbreaks at commercial farms causing extensive and prolonged morbidity and mortality. Of these pathogenic organisms, the ectoparasite monogenean *Dactylogyrus intermedius* is often in connection with epidemic occurred in significant freshwater-cultured cyprinid fish (Kritsky and Heckmann 2002). This species of parasite is commonly attached to the gills of fish from freshwaters and transmits principally by direct contact (Reed et al. 2009). Freshwater fish infected with *D. intermedius* would appear with clinical signs such as lethargy hypnody, appetite dwindles, even mucus excess, and ulcer (Alvarez-Pellitero 2004). The injurious effects of this parasite on host are also believed to cause secondary infection by bacteria and fungus, damage gill tissue, and disrupt respiratory system, resulting in growth retardation and high mortality, which introduce severe economic losses (Klinger and Floyd 2009; Tonguthai 1997).

The most conventional anthelmintics used to control *Dactylogyrus* are diverse bath treatments of infested fish, including praziquantel, toltrazuril, and mebendazole (Buchmann et al. 1993; Schmahl and Mehlhorn 1985; Schmahl et al. 1988). Some other chemicals such as trichlorfon, formalin, and new triazine derivative also have been evaluated for their therapeutic effect (Ellis 1974; Schmahl 1993). These parasiticides, nevertheless, have either been prohibited or have triggered considerable concerns because of threats of drug resistance, risk of drug residue, and toxicity to nontarget organisms due to frequent use (Burka et al. 1997; Yuan and Chen 2012). Therefore, there is increasing research on the natural anthelmintic resource. Chitwood (2002) reviewed that higher plants yield a broad spectrum of active

phytochemicals such as alkaloids, terpenes, and simple or complex phenolics displaying antagonistic toward plant–parasitic and other nematodes. The extracts of *Radix angelicae pubescentis*, *Semen pharbitidis*, *Bupleurum chinense* DC, etc. were reported effective in dislodgement and mortality of *D. intermedius* in our previous work (Liu et al. 2010; Wu et al. 2011).

Dryopteris crassirhizoma (Dryopteridaceae), named Mianma Guanzhong in Chinese (Chinese Pharmacopeia Commission 2005), is a semi-evergreen plant that is widely distributed in Japan, Korea, and China as a pteridophyte. The rhizomes of *D. crassirhizoma* are traditionally used as an herbal remedy for various diseases, such as flu, verminosis, bacterial disease, and cancer (Banerjee and Sen 1980; Chang et al. 2010). Much researches revealed that this plant possesses numerous pharmacological activities, including antioxidant, tumoricidal, and fatty acid synthase inhibitory activity (Lee et al. 2003; Mazzio and Soliman 2009; Na et al. 2006). Furthermore, bioactivities of *D. crassirhizoma* are attributed mainly to the presence of phloroglucinol, flavonoid, and other polyphenols (Gao et al. 2008). The vermifuge activity of *Dryopteris* against *Schistosoma mansoni* is related to the presence of phloroglucinol derivatives (Magalhaes et al. 2010), and withal, flavonoid glycosides naturally occurred in *D. crassirhizoma* exhibited antiinflammatory and antioxidant/pro-oxidant activity, inhibitory effects on human immunodeficiency virus-1 reverse transcriptase (Calderón-Montaño et al. 2011; Min et al. 2001).

Although a great number of researches have been conducted on the chemical composition and biological activities of phenol derivatives from *D. crassirhizoma*, there have been no reports on in vivo anthelmintic activity of isolated active compounds against *D. intermedius*, and this is precisely the purpose of this work.

Materials and methods

Plant materials

The root of *D. crassirhizoma* was purchased from a local herbal drug market (Xi'an, Shaanxi, China) in 2011. The voucher specimen was identified by Prof. X.L.He (Northwest A&F University, Shaanxi, China), and has been deposited in the herbarium of the college of Life Science, Northwest A&F University.

Plant extraction and fractionation of active principles

The dried rhizomes (4 kg) were ground to a coarse powder using mechanical pulverizer and ultrasonic extracted with methanol (MeOH) (10 L×4 times) at 60 °C for 40 min each time. The extract was filtered, concentrated under reduced pressure in a vacuum rotary evaporate, and desiccated to yield 254 g of methanol extract. The MeOH extract was then partitioned using petroleum ether (60–90 °C) and ethyl acetate (EtOAc). The concentrated EtOAc phase was submitted to silica gel CC using chloroform–methanol (gradient, 1:0→0:1, v/v) solvent mixtures and obtained three fractions (A–C). All fractions were monitored and combined based on thin-layer chromatography. Fraction C was further purified by recrystallization in chloroform–methanol and a pale yellow crystal (**3**) was obtained. Fraction A was subjected to column chromatography on silica gel and ODS-A (12 μm, YMC) in succession using chloroform–methanol (10:1) and water–methanol (8:2), respectively, to afford a compound (**1**). Compound (**2**) was isolated from fraction B as a yellowish powder by repeated silica gel CC using chloroform–methanol mixture as eluent.

Preparation of infected goldfish

Healthy goldfish (*Carassius auratus*), with a mean weight of 4.3±0.3 g, were obtained from a local fish farm (Xi'an, Shaanxi, China) as animal model and cultured in a 180-L glass aquarium under laboratory condition (water temperature 24±1 °C, oxygen content higher than 85 % saturation). Fish were acclimatized to culture condition for a period of 7 days and then were polycultured with the ones infected with *D. intermedius* which were maintained in our laboratory. According to the process described in our previous work (Wang et al. 2008), the infested fish were prepared following the procedure comprised of collecting eggs, hatching eggs, and reinfection of the parasites. After 3 weeks of cocultivation, five fish were randomly selected, killed by spinal severance, and eight branchial filaments of each fish were biopsied to determine the parasite infection level and intensity under a light microscope (Olympus BX41, Tokyo, Japan) at 10×4 magnification. Fish were chosen for the in vivo assay when the infection rate was 100 %, and the mean number of parasites on gills was about 30 per fish.

In vivo anthelmintic efficacy assay

The tests were conducted in 2-L plastic pot containing 1 L of constantly aerated working solutions and five previously infected fish. Water parameters were kept similar to culture condition above. Initial tests were undertaken to seek out a suitable concentration range for the efficacy assay in case of mortality of fish. The stock solutions were prepared by dissolving fractions and pure compounds in dimethyl sulfoxide (DMSO) at the concentration of 100 mg mL^{-1}. The desired concentrations of compounds were prepared by adding adequate amounts of stock solutions to 1 L of water. Control groups without any fractions or chemicals were set up under the same experimental conditions as the test groups.

Meanwhile, other control groups containing only DMSO were introduced to determine the possible effects of DMSO. Forty-eight hours after treatment, mortality of fish was recorded, and all the surviving fish were killed by spinal severance; lamella branchialis were biopsied under a light microscope to ascertain the number of parasites. The anthelmintic efficacy tests were conducted in triplicate, and the efficacies were confirmed by the comparison of the number of parasites in each treatment group with those in the control group, and calculated using the following equation:

$$AE(\%) = [B-T(P)]/B \times 100$$

In which AE is anthelmintic efficacy, B and T represent the average number of surviving *D. intermedius* in the blank control and treatment groups, respectively.

Acute toxicity

Acute toxicity test of the two compounds (**2** and **3**) was conducted for goldfish in a tank of 5.0 L capacity, containing 2-L test solution, and ten goldfish. Tests were conducted in duplicate, and the main measures include the water temperature at 24±1 °C, continuous aeration. The pure compounds were performed at a different series of concentrations from 10 to 50 mg L^{-1} in an equidifferent way. The death of fish was recorded when the opercula movement and the tail beat stopped, meantime; the fish no longer responded to mechanical stimulus. Control groups were set under the same test conditions without chemicals. To avoid the deterioration of water quality, the dead fish were removed from the water promptly. Fish mortalities were registered after 48 h.

Electron microscopic observation

D. intermedius were collected from the gills of goldfish which were treated with the isolated compound **3** at median effective concentration (EC_{50}) concentration and washed for four times in water to remove most of the mucus. The organisms were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (PBS, pH 7.2) for over 24 h at 4 °C and then adsorbed onto poly-L-lysine-coated glass substrate. After rinsing with 0.1 M PBS and dehydrated through a graded ethanol series to isoamyl acetate, worms were dried using liquid CO_2 in an EMS 850 critical point drying apparatus and coated with gold coater for 2 min. Finally, specimens were observed under S-3400N scanning electron microscope with accelerating voltage of 15 kV.

Statistical analysis

All data in this study were performed using the software Predictive Analytics Software Statistic v. 18.0. Probit analysis (Finney 1971) was used for the calculation of the median lethal concentration (LC_{50}) and EC_{50} values at the 95 % confidence interval.

Results

In vivo bioassay

In the present research, the dried underground parts of *D. crassirhizoma* were extracted with methanol for four times. The concentrated methanol extract was then partitioned with petroleum ether (ethyl acetate). Anthelmintic efficacy assay showed that the ethyl acetate fraction was active, and researches have shown that polyphenol derivatives in this plant were richly distributed in solvent of similar polarity to the ethyl acetate. Thus, it was further fractionated into three fractions on silica gel CC eluting with chloroform–methanol (1:0→0:1, v/v). Fraction A was separated by an ODS-C18 reverse-phase chromatography column to give a white crystal named compound **1**, which showed 89.8 % anthelmintic effect at the maximum experimental concentration up to 25 mg L^{-1}, and the EC_{90} could reach up to 27.44 mg L^{-1}. Compound **2** was obtained from fraction B by column chromatography on silica gel; EC_{50} and EC_{90} values for this compound were 3.01 and 6.08 mg L^{-1} (Table 1). Successive recrystallization of fraction C resulted in the isolation of compound **3**, and in anthelmintic efficacy assay, parasites were 100 % removed at the concentration of 12 mg L^{-1} (Fig. 1).

Table 1 Anthelmintic activity of the compounds **2** and **3** against *Dryopteris intermedius* and acute toxicity for goldfish after 48 h

Sample	Anthelmintic efficacy				Acute toxicity			
	EC_{50} (mg L^{-1}; 95 % CL)	EC_{90} (mg L^{-1}; 95 % CL)	χ^2	*P* value	LC_{50} (mg L^{-1}; 95 % CL)	LC_{90} (mg L^{-1}; 95 % CL)	χ^2	*P* value
Compound 2	3.01 (2.72–3.28)	6.08 (5.54–6.79)	2.22	0.70	36.22 (31.60–40.76)	46.01 (40.85–63.58)	1.09	0.78
Compound 3	2.71 (2.41–2.99)	5.84 (5.30–6.57)	3.47	0.48	32.47 (27.30–28.00)	47.31 (40.00–70.93)	1.88	0.60

EC_{50} 50 % effective concentration, LC_{50} 50 % lethal concentration, 95 %CL 95 % confidence limit, χ^2 chi-square

Both compounds **2** and **3** demonstrated obvious anthelmintic activity against *D. intermedius*, with compound **3** being the most active (EC_{50}=2.71 mg L^{-1}, EC_{90}= 5.84 mg L^{-1}).

Acute toxicity

The 48-h LC_{50} and LC_{90} values of the two active compounds (**2** and **3**) were evaluated using probit regression models which were given in Table 1. By the log concentration–probit equation, the calculated LC_{50} values were 36.22 and 32.47 mg L^{-1} for compounds **2** and **3**, respectively. Though compound **3** was slightly less likely to kill goldfish when their LC_{50} values are taken into consideration, fish mortality appeared within 48-h treatment of 12 mg L^{-1} of compound **3** that caused 100 % anthelmintic efficacy, but no fish were dead after bath treatment with compound **2** at the same concentration (Fig. 1). No mortality was observed in each blank control group.

Surface topography

The untreated control *D. intermedius* revealed a sleek body contour. Surface invaginations forming unapparent shallow corrugation are present throughout the body surface (Fig. 2a). By contrast, the helminths treated with compound **3** were seen to shrink sharply (Fig. 2b), and the tegument was extensively damaged with formation of intensive folds (Fig. 2c) and nodular-like protrusions (Fig. 2d).

Structural determination of active compounds

Compound **1**, $C_7H_6O_4$, was obtained as a white needle crystal. ITMS-ESI (negative mode): *m/z* 153.07 $[M–H]^-$; ^1H NMR (MeOD, 400 MHz) δ ppm: 6.82 (1H, d, *J*=8.4 Hz, H-5), 7.44 (1H, d, *J*=2.0 Hz H-6), 7.46 (1H, s, H-2); ^{13}C NMR (MeOD, 100 MHz) δ ppm: 170.39 (-COOH), 151.68 (C-4), 146.20 (C-3), 124.03 (C-6), 123.21 (C-1), 117.84 (C-5), 115.89 (C-2). These data were in agreement with the reported literature values. Thus, the structure of compound **1** was determined as protocatechuic acid (PCA) (Flamini et al. 2001).

Compound **2**, $C_{29}H_{32}O_{15}$, was isolated as a pale yellow powder. ITMS-ESI (negative mode): *m/z* 619.45 $[M–H]^-$; ^1H NMR (MeOD, 400 MHz) δ ppm: 0.79 (3H, d, *J*=6.3 Hz, Me-6″), 1.27 (3H, d, *J*=6.1 Hz, Me-6‴), 2.06 (3H, s, 4″-$COCH_3$), 3.51 (1H, m, H-5‴), 3.62 (1H, m, H-3‴), 3.90 (1H, m, H-3″), 4.03 (1H, dd, *J*=3.5, 1.8 Hz, H-2‴), 4.22 (1H, dd, *J*=3.4, 1.8 Hz, H-2″), 4.83 (1H, t, *J*=9.9 Hz, H-4″), 5.52 (1H, d, *J*= 1.8 Hz, H-1″), 5.56 (1H, d, *J*=1.9 Hz, H-1‴), 6.44 (1H, d, *J*= 2 Hz, H-6), 6.70 (1H, d, *J*=2.1 Hz, H-8), 6.94 (2H, d, *J*= 8.8 Hz, H-3′, 5′), 7.74 (2H, d, *J*=8.8 Hz, H-2′, 6′); ^{13}C NMR (MeOD, 100 MHz) δ ppm: 178.18 (C-4), 171.05 (4″-$\underline{C}OCH_3$), 162.14 (C-7), 161.57 (C-5), 160.47 (C-4′), 158.52 (C-2), 156.65 (C-9), 134.42 (C-3), 130.64 (C-2′, -6′), 120.89 (C-1′), 115.16 (C-3′, -5′), 106.13 (C-10), 101.16 (C-1″), 99.21 (C-6), 98.44 (C-1‴), 94.24 (C-8), 73.48 (C-4"), 72.20 (C-4‴), 70.65 (C-3‴), 70.31 (C-5‴), 70.29 (C-2″), 69.90 (C-2‴), 68.62 (C-5"), 68.24 (C-3″), 19.58 ($CO\underline{C}H_3$), 16.71 (C-6‴), 16.18 (C-6″). According to the reported literature values (Mizuno et al. 1991; Wu et al. 2012), the compound was identified as kaempferol 3-a-L-(4-O-acetyl)rhamnopyranoside-7-a-L-rhamnopyranoside (sutchuenoside A, SA).

Compound **3**, $C_{27}H_{30}O_{14}$, was obtained as a pale yellow crystal. ITMS-ESI (negative mode): *m/z* 577.45 $[M–H]^-$; ^1H NMR (DMSO-d_6, 400 MHz) δ ppm: 0.82 (3H, d, *J*=5.5 Hz, Me-6″), 1.14 (3H, d, *J*=6.1 Hz, Me-6‴), 3.11–3.20 (2H, m, H-4″, -5″), 3.30 (1H, m, H-4‴), 3.40–3.53 (2H, m, H-3‴,-5‴), 3.64 (1H, m, H-3″), 3.85 (1H, bars, H-2‴), 3.99 (1H, bars, H-2″), 4.63 (1H, d, *J*=5.9 Hz, OH-4″), 4.74 (1H, d, *J*=4.4 Hz, OH-3″), 4.78 (1H, d, *J*=5.9 Hz, OH-3‴), 4.90 (1H, d, *J*= 5.7 Hz, OH-4‴), 4.97 (1H, d, *J*=4.4 Hz, OH-2‴), 5.13 (1H, d, *J*=4.5 Hz, OH-2″), 5.31 (1H, d, *J*=1.4 Hz, H-1″), 5.55 (1H, d, *J*=1.4 Hz, H-1‴), 6.46 (1H, d, *J*1=12.1 Hz, H-6), 6.79 (1H, d, *J*1=12.1 Hz, H-8), 6.93 (2H, d, *J*1=18.8 Hz, H-3′, -5′), 7.80 (2H, d, *J*1=18.8 Hz, H-2′, -6′), 10.25 (1H, s, OH-4′), 12.61 (1H, s, OH-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ ppm: 177.93 (C-4), 161.70 (C-7), 160.93 (C-5), 160.15 (C-4′),

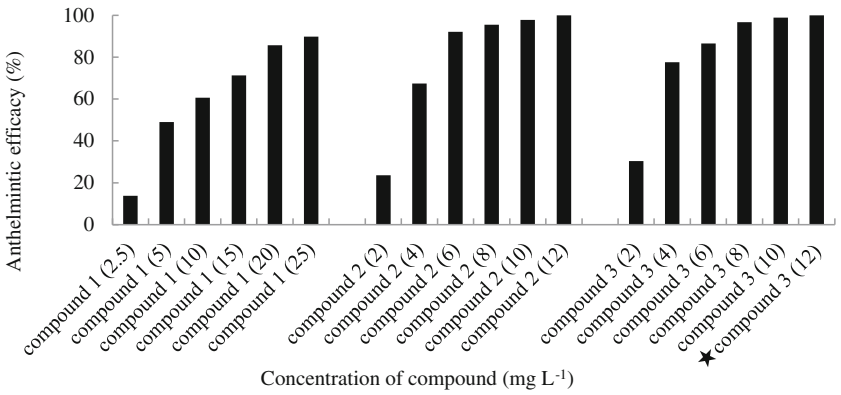


Fig. 1 Anthelmintic efficacy of compound **1**-**3** against *Dryopteris crassirhizoma* after 48 h of exposure. *Black star* indicates fish mortality occurred

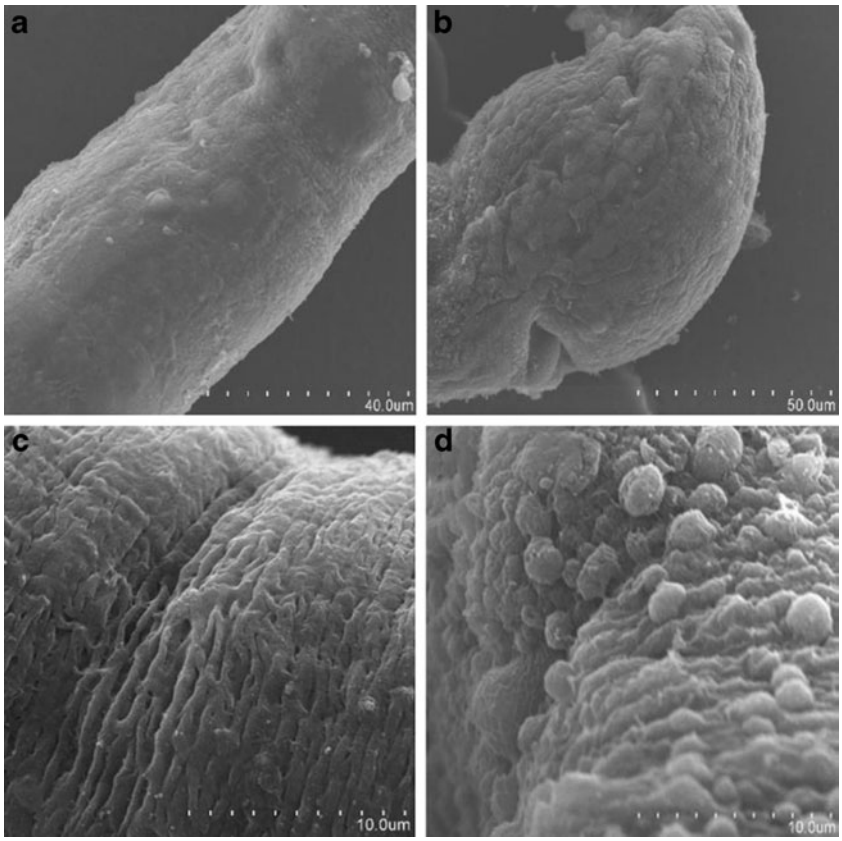


Fig. 2 *Dactylogyrus intermedius* scanning electron micrographs. **a** Untreated control helminth, showing smooth surface (*scale bar* = 40 μm). **b**, **c**, **d** SEM of parasite treated with compound, showing forms of disruption of tegumental surface. **b** Distinct contraction of body surface (*scale bar* = 50 μm), **c** view of ventral surface showing abnormal tegumental folds (*scale bar* = 10 μm), and **d** clumps of disheveled protuberances (*scale bar* = 10 μm)

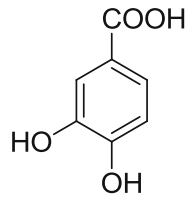
157.77 (C-2), 156.09 (C-9), 134.54 (C-3), 130.69 (C-2′, -6′), 120.35 (C-1′), 115.42 (C-3′, C-5′), 105.79 (C-10), 101.88 (C-1″), 99.45 (C-6), 98.44 (C-1‴), 94.59 (C-8), 71.59 (C-4″), 71.12 (C-4‴), 70.66 (C-3″), 70.33 (C-3‴), 70.24 (C-2″), 70.08 (C-2‴), 70.06 (C-5″), 69.80 (C-5‴), 17.89 (C-6‴), 17.45 (C-6″). Based on the above spectral data, the structure of compound **3** was assigned as kaempferol-3, 7-O-α-L-dirhamnoside (kaempferitrin, KM) which was consistent with the reported literature values (Chaturvedula and Prakash 2011; Gohara and Elmazar 1997).

The chemical structures of three compounds isolated from ethyl acetate extract of *D. crassirhizoma* are shown in Fig. 3.

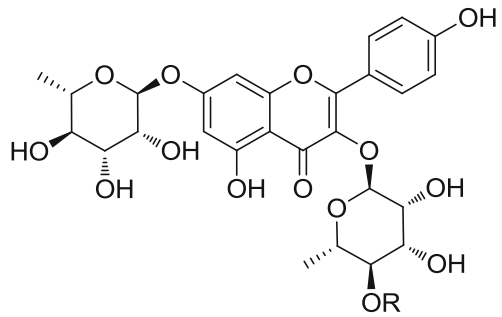
Discussion

Plants have been used for their medicinal properties all through our history. Although antibiotics had declined their usage and dwindled interest in basic research into their effects, the side effects of using antibiotic and other synthetic compound on safety and quality, as well as the risk to human of chemical residues and of drug tolerance being passed on to pathogens, have rejuvenated interest in developing plant resources as natural alternatives. This is illustrated quite strikingly in the case of seeking treatment for parasite infestation of fish. Early studies showed that ethanol extract of *Artemisia annua* was effective in the dislodgement and mortality of monogenean parasites of juvenile *Heterobranchus longifilis* and the leaf extracts of Indian almond can significantly decrease the number of *Gyrodactylus* and *Dactylogyrus* infection of gold fish, respectively (Ekanem and Brisibe 2010; Chansue and Tangtrongpiros 2005). Our previous work on the screening of medicinal plants for the anthelmintic activity against *D. intermedius*, monogenean, resulted in selection of *D. crassirhizoma* (Lu et al. 2012).

In the present study, three compounds, PCA (**1**), SA (**2**), and KM (**3**), were isolated from the rhizome of *D. crassirhizoma* through the use of chromatographic techniques. Thereamong, SA and KM displayed relatively good anthelmintic activity against *D. intermedius* in goldfish (*C. auratus*) as compared with PCA (EC_{50}=6.64 mg L^{-1}), with EC_{50} values of 3.01 and 2.71 mg L^{-1} after 48 h. Their application led to the reduction of the parasite load and even eliminated the parasites completely in some cases within the duration of exposure, and the two compounds were much more effective than ethyl acetate extract (EC_{50} value = 67.67 mg L^{-1}) (Lu et al. 2012), which suggested that SA and KM may be responsible for the anthelmintic activity of the ethyl acetate extract. In addition, a dose-dependent efficacy was observed in treated helminthes as an increase in concentration caused a more pronounced dislodgement effect in comparison with the blank control group. From the



protocatechuic acid (1)



R=CH_3CO- sutchuenoside A (2)

R=H kaempferitrin (3)

Fig. 3 Chemical structures of compounds isolated from *Dryopteris crassirhizoma*

observation under SEM in this study, KM induced pronounced tegumental damage and disruption in the soft-bodied platyhelminth parasites; the pathological effects include intensive wrinkles, holes, along with nodular structures. Similar to the present observations, the tegument surface was found to be a vital target organ for different synthetic drugs and natural anthelmintic products (Martin et al. 1997; Kundu et al. 2012). Thus, on assessment of the survivability and scanning electron micrographs, it becomes apparent that the active compound indeed possessed anthelmintic property against the test parasite.

The major previous researches on bioactivity of *D. crassirhizoma* have been centered on its rhizome, and the identified essential components include plastocyanin, triterpene, phloroglucinol, and flavonoid (Dennison et al. 2001; Shiojima et al. 1994; Widén et al. 1975; Zhang et al. 2012). Plastocyanin has been used for studies of electron transfer in many biological processes because of its unique structure (Pletneva et al. 2000). It has been reported that triterpenes possessed potent inhibitory activity against HIV-1 protease (Lee e¯t al. 2008). Studies of Kapadia et al. (1996) and Lee et al. (2009) have shown that phloroglucinol derivatives were linked to antitumor promoting and antibacterial activity of the rhizome of *D. crassirhizoma*.

Flavonoids are prominent naturally occurring phenolic secondary metabolites, and are generally present as glycosides in medicinal plants, less frequently aglycones (Beecher 2003; Harborne and Williams 2000). Besides a series of medicinal properties, such as antimicrobial activity and vascular activity (Cushnie and Lamb 2005; Xu et al. 2007), flavonoids are implicated in anthelmintic effects (Barrau et al. 2005; Zahir et al. 2012). It is presumed that flavonoid and condensed tannins, polymers of flavonoid units, exert action due to the possibility of activity on certain enzymes such as esterases and neuronal nitric oxide synthase, or the ability to bind to glycoprotein on the cuticle of the parasite (Kar et al. 2002; Thompson and Geary 1995). However, to the best of our knowledge, this is the first report on this sort of compound about its anthelmintic effect against *D. intermedius*. SA and KF are two of flavonol glycosides acquired from nature that possess a wide range of activities. Wu et al. (2012) recently reported analysis process and the total assignment of ^{1}H and ^{13}C signals of SA in detail on the basis of various one-dimensional and two-dimensional NMR experiments. Early study of Kwon et al. (2004) demonstrated that SA inhibits significantly lipopolysaccharide-induced nitric oxide production in a concentration-dependent manner. The other compound KF, a 3,7-diglycosylflavone, is produced by several plants, exhibited a strong antioxidant potential preventing the in vitro lipid peroxidation in different lipid bilayers, and led to a dose-related hypotension in genetically prone hypertensive rats (de Sousa et al. 2004; Gohara and Elmazar 1997). This compound also was able to induce immunostimulatory effects on immune responses mediated by splenocytes, macrophages, PBMC, and NK cells and induce high cytotoxic and antitumor effects against HeLa cells (Alonso-Castro et al. 2013; Del Carmen et al. 2013). Recently, an area of research that is of particular interest is the energy generation process. Vishnu Prasad et al. (2009) showed that KM inhibits glucose uptake in adipocytes by decreasing GLUT4 translocation and directly interacting with GLUT4, thereby competing with glucose for the transport. The investigation of flavonoid on rat revealed that oxidative phosphorylation in cardiac mitochondria is partially uncoupled, and the capacity of mitochondria to synthesize ATP diminished (Trumbeckaite et al. 2006). Several citrus flavonoids were found to inhibit glycogen synthase kinase-3β activity, and GSK-3 has been confirmed to be a potential drug target for the treatment of parasitic disease African trypanosomiasis (Johnson et al. 2011; Ojo et al. 2008). Consequently, the antiparasitic effect of SA and KM may be relevant to interference in energetic processes, but further analyses of the precise mode of action involved and measurements of phenolic compounds like flavonoid glycosides as well as flavonoid aglycones are required in details for delineation of their therapeutic efficacy.

In order to evaluate the acute toxicity to host, the two active compounds were tested at concentrations which caused different levels of mortality of goldfish. The results showed that these two isolated chemicals are safe for host because the 48-h LC_{50} values are almost 12 times the effective ones, and there was hardly any death of the host at median effective dose. In

addition, the safety of hydrolysate kaempferol for human health has been generally recognized, and it has been regulated as an approved food additive in several nations (Lim et al. 2007). Therefore, in combination with their acceptable activity, SA and KM are very attractive in the treatment of *D. intermedius*.

In conclusion, our results indicate that kaempferol rhamnosides SA and KM, isolated from the rhizomes of *D. crassirhizoma*, possess satisfactory in vivo anthelmintic activity against *D. intermedius*. Also, both compounds are of high safety for the host with the median lethal concentration at a high level. Finally, as shown in the present study, the flavonol derivatives could be considered as a promising class for the development of new agent against *D. intermedius*, yet specific mechanism and larger-scale field evaluation are necessary to be further explored.

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