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In vitro immunocompetence of two compounds isolated from *Polygala tenuifolia* and development of resistance against grass carp reovirus (GCRV) and *Dactylogyrus intermedius* in respective host

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ABSTRACT

The present study was undertaken to isolate some compounds from methanol extract of *Polygala tenuifolia* and evaluate their immunostimulatory properties and antiviral activity using grass carp *Ctenopharyngodon idella* kidney (CIK) cells and GCRV. By applying insecticidal bioassay-guided, chromatography techniques and successive recrystallization, two purified compounds were obtained. The changes of expression of selected immune genes (Mx1, IL-1β, TNFα, MyD88 and IgM) in *C. idella* kidney cell lines were evaluated after exposure to these isolated compounds. The results showed that compound 1 and 2 up-regulated to varying degrees of Mx1, IL-1β, TNFα, and MyD88 in *C. idella* kidney cells. WST-8 kit assay verified the two compounds has no toxic effects on CIK cell, and furthermore, have *in vitro* antivirus activity. Especially, that there is keeping 79% cell viability when exposure to compound 2 (100 mg L^{-1}). According to *in vivo* insecticidal assays against *Dactylogyrus intermedius*, compound 2 exhibited higher efficacy than compound 1, which was found to be 87.2% effective at the concentrations of 5 mg L^{-1} and safe to goldfish (*Carassius auratus*). Besides, the purified compounds were identified by spectral data as: (1) 1,5-Anhydro-D-glucitol and (2) 3,4,5-trimethoxy cinnamic acid. Overall, the results indicate that bath administration of these compounds modulates the immune related genes in *C. idella* kidney cells and to some extent, eliminate the virus and parasitic infections.

1. Introduction

Over the past half a century the aquaculture production has grown dramatically to around 52.5 million tonnes in 2008 worth US$98.5 billion [1]. Despite of the huge success, the sector faces important challenges with respect to controlling variety of diseases. Parasite, pathogenic bacteria, fungus and virus as main pathogenic factor cause mortality occurred and recurrent outbreaks in aquaculture, especially in large-scale aquaculture ponds [2–4]. In response, antibiotics had been widely used as therapeutic agent to prevent these infections in aquaculture but accompanied by some practices potentially damaging to human and animal health [5,6]. In recent years, many researchers have been researching and exploiting effective and environment safe compound to replace antibiotics [7]. However, increasing resistance of the fish to a specific pathogen does not necessarily protect them from other pathogens. Enhancement of the innate immunity of fish on the other hand may provide broad-spectrum resistance to infections [8]. Application of immunostimulants to activate or enhance the innate immune system has been widely accepted as a good alternative to improve health of cultured fish and resist disease [9–11].

A large number of plants as environmentally friendly materials have been used in traditional medicine for the treatment and control of diseases [12–14]. Four of such plants are *Zingiber officinale*, *Dryopteris crassirhizoma*, *Kochia scoparia*, and *Polygala tenuifolia*. On the other hand, some of the medicinal plants were found to have immune function and have been used as the immunostimulants for fish [12,15]. Furthermore, fewer botanical components, including diterpenes andrographolide and neoandrographolide from *Andrographis paniculata* [16], have been known as potent immunostimulants. But it has been lack of the immune related genes research of botanical components [16,17] compared with the bacterial components [18]. These components do not exist in host cells and are called pathogen-associated molecular patterns (PAMPs). To recognize PAMPs, vertebrate hosts have soluble and

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membrane-associated pattern-recognition receptors and one of the most important families of cellular receptors are the toll-like receptors (TLRs) [19]. Individual TLRs and antigen receptors immunoglobulin (IgM) play important roles in recognizing specific components derived from pathogens [20–22]. The components trigger MyD88-dependent TLRs signalling cascades, and enable the host to mount an effective innate immune response through the induction of cytokines such as interleukin-1β (IL-1β), tumour growth factor-a (TNF-α), interferon-regulated myxovirus resistance locus (Mx1) and so on [23,24].

In such cases, myeloid differentiation factor 88 (MyD88), an important adaptor molecule in TLR signalling pathway, plays an important role in the host defence against bacterial infections as MyD88-deficient mice did not produce any detectable levels of cytokines in response to *Staphylococcus aureus* and showed high mortality after infection [25]. Cytokines are small molecular weight proteins and include interferons (IFN), interleukins (IL), chemokines, monokines and certain growth factors [17]. Among these proteins, both TNF-α and IL-1β are cytokines that induce or facilitate inflammatory response. IL-1β adjusts immune responses to various pathogens and plays an important role in host response to microbial invasion, tissue injury and immunological reactions including autoimmune diseases, while TNF-α is crucial for diverse cellular responses, including cell proliferation, differentiation and the induction of other cytokines [11,26]. Moreover, Mx pathway is one of the most powerful pathway among the known IFN-induced antiviral mechanisms, which discovered large inter-specific variability Mx (from 1 to 7) isoforms in teleost. Among them, Mx1 as a key component of the antiviral state showed a largest ability to protect against GCRV than Mx2 and Mx3 [27]. IgM constitute the major component of the natural antibodies and is the first class of antibodies produced during a primary antibody response [28]. On the other hand, IgM provides the response to antigen and plays a regulatory function in subsequent immune response development, promoting the production of high affinity IgG [20–22]. Besides, the antigen receptor IgM and Toll-like receptors could synergetic activate B cells, such as activation of rheumatoid factor $(RF)^+$ B cells [22].

The GCRV has been known in China since 1983 and now have identified many strains, including the published whole-genome sequences strain GCRV 873, HZ08, GD108, and 104 and so on [29]. This disease caused severe hemorrhagic problems in grass carp, affecting about 85% mortality in fingerling and yearling grass carp [29]. For potential antivirus mechanism, myxovirus resistance (Mx) proteins are crucial effectors of the innate antiviral response against a wide range of viruses, mediated by the type I interferon signalling pathway. The genus *Dactylogyrus* is one of the most parasitic disease on the gills of various species, including grass carp (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), big head carp (*Hypophthalmichthys nobilis*) and black carp (*Myelopharyngodon piceus*) and so on [13,30,31]. The fishes infected with *Dactylogyrus* can be seriously damaged such as loss of appetite, slow-growing and high mortalities [32]. In recent years, some compounds were found to effective against *Dactylogyrus intermedius*, including steroidal saponins, osthol, isopimpinellin, arctigenin, arctiin and so on [31,33,34].

P. tenuifolia as a natural agent, has been used in the treatment of various disease, including anti-tumour [35], sedative effect [36], insecticidal activity [13], anti-inflammation [37], neurite outgrowth [38] and so on. Root extract of *P. tenuifolia* have the activity against *D. intermedius* in goldfish (*C. auratus*), as reported by our previous research [13]. However, it is un-known the nosotropic component and the immunologic function of *P. tenuifolia* on fish. This study was undertaken to research the disease-resistant activity of the compound isolated from the *P. tenuifolia* and examine if isolated compound would influence immune-related gene expression in *C. idella* Kidney cells including induced MyD88, IL-1β, TNF-α, Mx1 and IgM. Additionally, the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophe- nyl)-2H tetrazolium, monosodium salt (WST-8) assay was performed to analyse the viability of the cells stimulated by the isolated compounds.

2. Materials and methods

2.1. Isolation of compounds from P. tenuifolia Willd

P. tenuifolia Willd were collected in Shaan'xi province, China, in June, 2012. The 2000 g plant was air-dried under sunlight for a week and finally oven-dried at 45 °C for 48 h, then crushed and screened with 40–60 mesh stainless steel sieve. The powdered sample was freeze-dried at 40 °C over-night and then extracted with methanol in water bath at 60 °C for 3 h. The extract of *P. tenuifolia* was filtered and concentrated using a rotary vacuum evaporator and added aether 1:3 (v/v) then filtering removed the precipitation. The filtrate mixed with water-saturated n-butyl alcohol 1:1 (v/v) for 2 h, then removed the aqueous phase and volatilized the solvent yielding 272 g n-butyl alcohol extract (BE). Part of the extract (240 g) was fractioned to column chromatography (1200 mm × 100 mm) using silica gel (2000 g, 100–200 mesh) and eluted with dichloromethane: methanol gradient to provide 410 fractions (400 mL each). These fractions were combined to give six main fractions (Fr. A: 1–86, Fr. B: 87–147, Fr. C: 148–211, Fr. D: 212–348, Fr. E: 349–400) based on TLC analysis. The Fr. D (144 g) was found to have higher anthelmintic activity than others and part of the fraction (90 g) was subjected to further isolation using silica gel (200–300 mesh) to give 182 fractions (300 mL each). These fractions were combined to give four main fractions (Fr. Da: 1–31, Db. B: 32–88, Dc. C: 89–166, Fr. Dd: 167–182) based on TLC analysis. The Fr. Dc (45 g) was found to have higher anthelmintic activity and part of the fraction (8.0 g) was isolated and eluted with n-butyl alcohol: ethyl acetate: water (4:1:5). Then dig out the adsorbent (guided by uv lamp irradiation) before the eluent reached the bottom. Finally, two pure compounds were obtained from the isolated adsorbent by aqueous extract and successive crystallization.

2.2. Cell culture and treatment

C. idella kidney (CIK) cells, provided by Prof. Ling-bing Zeng, Yangtze River Fisheries Research Institute Wuhan, Hubei, China, were cultured in sterile 6-well tissue culture plates containing 5.0 ml MEM media supplemented with 10% inactivated foetal calf serum (GIBCO/BRL) and maintained in incubator at 28 °C. For isolated compounds treatment, unilaminar cells (about 24 h after subculture cell) were treated with compound 1 and 2. Compounds were tested at three different concentrations added a control group, i.e. 0, 1, 10 and 100 μg mL^{-1}. For kinetic studies, the sampling times of treated cells were choosing 2, 8, 24 and 48 h post-stimulation and RNA was extracted and reversely transcribed. Each treatment was prepared in triplicate.

2.3. RNA extraction and reverse transcription

Total RNA was extracted from CIK cells using TRIzon Reagent (CWBIO, Beijing. PR China) following the manufacturer's instructions. The concentration of the RNA in each sample was tested on a 1% agarose gel and quantified spectrophotometrically (GeneQuant Pharmacia Biotech, Hørsholm, Denmark) and the quality determined as the OD260 nm/OD280 nm ratio with expected