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NOTE

In vitro effects of the polyphenols resveratrol, mangiferin and (–)-epigallocatechin-3-gallate on the scuticociliate fish pathogen *Philasterides dicentrarchi*

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ABSTRACT: This study investigated the *in vitro* effects of the polyphenols resveratrol, mangiferin and (–)-epigallocatechin-3-gallate (EGCG) on the histiophagous ciliate *Philasterides dicentrarchi*, which causes fatal scuticociliatosis in farmed turbot *Scophthalmus maximus* L. Of the 3 polyphenols, resveratrol showed strongest antiprotozoal activity, reducing ciliate density after 1 wk culture by, on average, 91% at 50 µM, and 96% at 500 µM. EGCG reduced ciliate density by, on average, 93% at 500 µM, with no significant effect at 50 µM. Mangiferin reduced ciliate density by, on average, 56% at 500 µM, again with no significant effect at 50 µM. In view of these findings, we discuss the potential utility of chemotherapy with polyphenols as a strategy for the control of scuticociliatosis in farmed turbot.

KEY WORDS: Polyphenols · *Philasterides dicentrarchi* · Resveratrol · Mangiferin · Epigallocatechin

INTRODUCTION

The histiophagous scuticociliate *Philasterides dicentrarchi* is an emergent infectious agent that causes important economic losses in farmed turbot (Iglesias et al. 2001). It can be readily eradicated from an external environment; however, once the ciliate has entered the body of the fish this is no longer possible (Paramá et al. 2003), and there is at present no effective treatment for endoparasitic infection by *P. dicentrarchi*. Chemotherapeutic measures for the control of scuticociliatosis in farmed turbot currently present 3 major problems: (1) *P. dicentrarchi* is a highly virulent species that divides rapidly and invades many organs (Iglesias et al. 2001); (2) at present, few chemotherapeutic agents are approved for use in aquaculture; and (3) some of the most effective compounds against *P. dicentrarchi* show reduced effectiveness in seawater (Iglesias et al. 2002). There is currently increasing interest in the therapeutic use of plant polyphenols to prevent pathological effects arising from the overproduction of oxygen and nitrogen radicals produced during oxidative stress (Young & Woodside 2001). The polyphenols mangiferin and (–)-epigallocatechin-3-gallate (EGCG) have additionally been shown to have potent effects against protozoan and helminth parasites infecting mammals (Das et al. 1989, Awe et al. 1998, Kolodziej et al. 2001, García et al. 2003).

Here we report a preliminary *in vitro* study of the effects of mangiferin, EGCG and a third plant polyphenol, resveratrol, on *Philasterides dicentrarchi*. Mangiferin (MA) is a xanthone C-glycoside obtained from the mango *Mangifera indica*; EGCG is a proanthocyanidine (condensed tannin) present, for example, in tea *Camellia sinensis* and wine from *Vitis vinifera*; resveratrol (RESV, 3,5,4′-trihydroxystilbene) is a phy-

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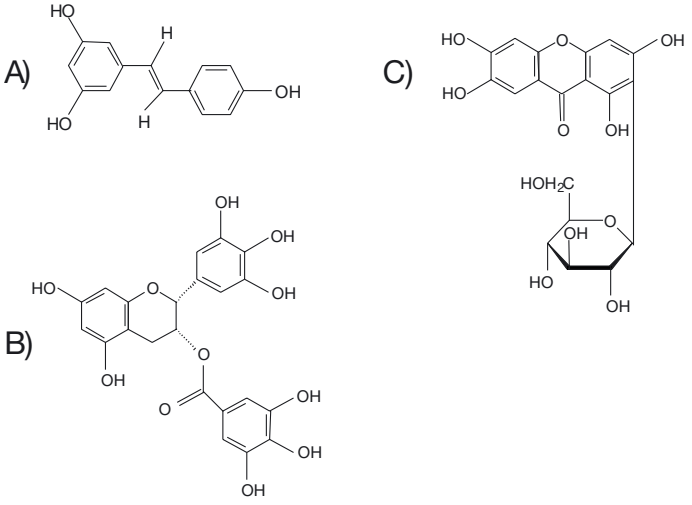

Fig. 1. *Philasterides dicentrarchi.* Chemical structures of (A) *trans*-resveratrol (RESV), (B) the polyphenolic flavonoid (–)-epigallocatechin-3-gallate (EGCG) and (C) the xanthone C-glycoside mangiferin (MA)

toalexin (phytoestrogen) synthesized by *Vitis vinifera* in response to injury or fungal attack, and is present at high concentrations in grape skin and red wine.

MATERIALS AND METHODS

The scuticociliate *Philasterides dicentrarchi* was obtained from ascitic fluid from the body cavity of naturally infected turbot *Scophthalmus maximus*, and maintained in complete L-15 medium (Leibovitz, Sigma) under the culture conditions previously described (Iglesias et al. 2003), which maintain the capacity of the parasite to infect turbot (Iglesias et al. 2002). The polyphenols *trans*-resveratrol (RESV), mangiferin (MA) and (–)-epigallocatechin-3-gallate (EGCG) (Fig. 1) were purchased from Sigma (Sigma Química) and maintained as 100 mM stock solutions in dimethyl sulfoxide (DMSO) at –20°C in the dark until use. To investigate antiprotozoal effects, the polyphenols (RESV, MA, or EGCG) in 1 ml of complete L-15 medium were added to the wells of 24-well sterile culture plates (Corning) containing 5×10^3 ciliates per well, to a final concentration of 50 or 500 µM, with subsequent incubation for 1 wk at 16°C. After this incubation period, the number of ciliates present was quantified in a 25 µl aliquot from each well: the ciliates were first inactivated with glutaraldehyde (final concentration 0.25%) for 15 min at room temperature, then counted in a hemocytometer (Iglesias et al. 2002). All experiments were done in quintuplicate. To rule out possible effects of the solvent DMSO, quintuplicate wells with L-15 medium containing the highest DMSO concentration used (0.5%) were also included. The results are expressed as means ± standard error (SEM), and statistical significances were assessed by 1-way ANOVA followed by Tukey-Kramer tests for multiple comparisons.

RESULTS AND DISCUSSION

The effects of RESV, MA, and EGCG (50 or 500 µM) on the *Philasterides dicentrarchi* trophozoite count in culture after 1 wk incubation are summarized in Fig. 2. As can be seen, the solvent DMSO had no significant effect; in previous studies we have found that DMSO at up to 2.5% does not affect *P. dicentrarchi* viability (Iglesias et al. 2002), and in the present study the maximum concentration used was 0.5%. The polyphenol with strongest antiprotozoal effect was REV, which reduced the trophozoite count (with respect to control wells) by, on average, 91% at 50 µM; by contrast, MA and EGCG only reduced the count significantly at 500 µM, by, on average, 56 and 93% respectively. Other plant polyphenols have previously been shown to have antiparasitic activity: for example, natural perotins (phenolic bisbibenzyiethers) and diphenyl ethers have

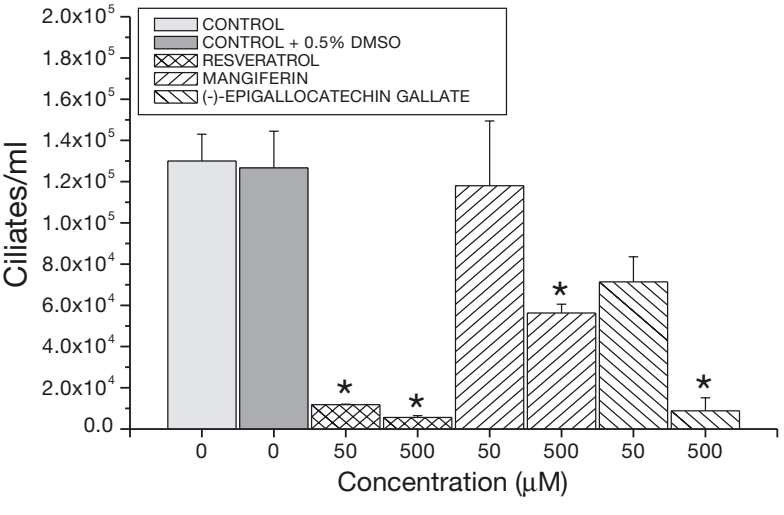

Fig. 2. *Philasterides dicentrarchi. In vitro* effects of resveratrol, mangiferin and (–)-epigallocatechin-3-gallate (50 or 500 µM) on viability of trophozoites. Each bar shows the mean estimated number of ciliates per well (±SEM, n = 5 wells) after 1 wk incubation with the polyphenol (initial number of ciliates per well: 5×10^3). Asterisks indicate a significant difference ($p < 0.01$) with respect to the corresponding control value

been reported to have nematocidal effects on *Caenorhabditis elegans* and *Nippostrongylus brasiliensis* (Gamenara et al. 2001), while MA has been reported to have *in vivo* inhibitory effects on embryogenesis in the nematode *Trichinella spiralis* (García et al. 2003). Likewise, several hydrolyzable tannins have been shown to have antiprotozoal activity against intracellular amastigotes of *Leishmania donovani* (Kiderlen et al. 2001, Kolodziej et al. 2001). Type-A proanthocyanidins, especially EGCG, are potent antiamoebic and antigiardial agents *in vitro* (Calzada et al. 1999a,b, Meckes et al. 1999). MA shows antibacterial activity *in vivo* (Calzada et al. 1999a,b, Meckes et al. 1999), though its antiprotozoal activity has not been investigated previously. RESV is a phytoalexin involved in grapevine resistance to microbial infection (Jeandet et al. 2002), and protects the plant from infection by *Botrytis cinerea* (Schouten et al. 2002) and other phytopathogenic fungi (Celimene et al. 2001). Several studies have demonstrated its activity against bacterial and fungal skin infections in humans (Chan 2002), but there have again been no previous studies of its antiprotozoal activity.

The present results thus indicate that RESV, and to a lesser extent MA and EGCG, have antiprotozoal activity against *Philasterides dicentrarchi* trophozoites *in vitro*. This raises the possibility of *in vivo* treatment of fish, probably by inclusion of the polyphenol(s) in the feed, which avoids chemical alterations such as may occur when they are administered in seawater baths (Iglesias et al. 2002). Clearly, it first needs to be investigated whether the *in vitro* antiprotozoal effects are maintained *in vivo* after oral administration. Certainly, these polyphenols have low toxicity even at very high doses (García et al. 2003), and can thus be administered over long periods. In addition, and as is well known, polyphenols are antioxidant agents that protect the tissues against oxidative stress, and human consumption of antioxidants has many alleged health benefits, including protection against cardiovascular diseases, and, most recently, cancer (López-Vélez et al. 2003). During *P. dicentrarchi* infection, there is an intense inflammatory cell response (principally monocytes/macrophages and lymphocytes; Iglesias et al. 2001). As we have observed previously, this response may be attenuated by these polyphenols (Álvarez et al. 2002, García et al. 2002, Leiro et al. 2002, 2003), with consequent reduction of associated immunopathological effects. Thus the plant polyphenols considered in the present study, notably RESV, are of potential interest in view of both possible antiprotozoal effects against *P. dicentrarchi* and possible anti-inflammatory effects during *P. dicentrarchi* infection. These polyphenols might be obtained naturally, or might be alternatively be used as chemical models for the design of new synthetic antiprotozoals.

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