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Citation for published version:
Burt, SA, Tersteeg-Zijderveld, MHG, Jongerius-Gortemaker, BGM, Vervelde, L & Vernooij, JCM 2013, 'In vitro inhibition of Eimeria tenella invasion of epithelial cells by phytochemicals' Veterinary Parasitology, vol 191, no. 3-4, pp. 374-378. DOI: 10.1016/j.vetpar.2012.09.001

Digital Object Identifier (DOI):
10.1016/j.vetpar.2012.09.001

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Veterinary Parasitology

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Download date: 28. Apr. 2017

Veterinary Parasitology 191 (2013) 374–378

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

In vitro inhibition of *Eimeria tenella* invasion of epithelial cells by phytochemicals

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ARTICLE INFO

Article history:
Received 14 December 2011
Received in revised form 29 August 2012
Accepted 2 September 2012

Keywords:
Eimeria tenella
Carvacrol
Curcumin
Echinacea purpurea
Betaine
MDBK

ABSTRACT

Resistance to coccidiostats and possible future restrictions on their use raise the need for alternative methods of reducing coccidiosis in poultry. The aim of this study was to evaluate the effect of selected phytochemicals on *Eimeria tenella* sporozoite invasion in vitro. Four phytochemicals were selected on the basis that they reduce the virulence of *Eimeria* spp. and/or provide immune modulatory benefits to host cells: betaine, carvacrol, curcumin and *Echinacea purpurea* extract (EP).

Madin–Darby bovine kidney (MDBK) cells were covered by medium containing phytochemicals at the highest concentration which was non-toxic to the cells. Salinomycin 50 μg/ml was positive control; negative control was medium only. *E. tenella* (Houghton strain) sporozoites were added to wells and after incubation for 2, 4 or 20 h at 37 °C, cells were fixed and stained with hematoxylin–eosin. Ten evenly spaced fields per well were photographed and the percentage of cells invaded by sporozoites was calculated and normalized to the control.

At 2 h, carvacrol, curcumin and EP showed a significantly lower percentage of sporozoite invasion than the untreated control; in contrast, betaine treatment represented a significantly higher invasion percentage. Combining carvacrol with EP inhibited *E. tenella* invasion more effectively than applying the compounds individually, but the further addition of curcumin did not reduce invasion further.

In conclusion, this study shows that invasion of MDBK epithelial cells by *E. tenella* sporozoites is inhibited in the presence of carvacrol, curcumin, or EP and enhanced by betaine. There may be potential for developing these phytochemicals as anti-coccidial feed or water additives for poultry.

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1. Introduction

Eimeria tenella is an economically important intestinal parasite of poultry that causes coccidiosis characterized by enteritis. Although laying hens generally recover from the illness and become immune, global financial losses due to coccidiosis in broiler flocks are estimated to be in excess of $3 billion annually (Dalloul and Lillehoj, 2006). The addition of coccidostats to poultry feed can control the disease, but the emergence of drug resistance as well as a possible future ban restricting coccidiostat use mean that there is an urgent need for alternative methods of reducing the burden of disease in poultry (Peek and Landman, 2003).

Four phytochemicals were selected on the basis that they reduce the virulence of *Eimeria* spp. and/or provide immune modulatory benefits to host cells (Peek, 2010).

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0304-4017/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetpar.2012.09.001

Betaine may have a stabilizing and protective effect on host cells (Virtanen, 1995). Carvacrol is a constituent of oregano (Giannenas et al., 2003); curcumin is an anti-inflammatory constituent of turmeric and has been reviewed recently (Kholi et al., 2005). *Echinacea purpurea* (purple cone flower) extract (EP), has anticoccidial properties which are attributed to immune modulation (Stimple et al., 1984).

The aim of this study was to evaluate the effect of betaine, carvacrol, curcumin and EP extract on the invasion of epithelial cells by *E. tenella* sporozoites in vitro. Combinations of the most inhibitive compounds were also tested.

2. Materials and methods

Carvacrol (98% pure) and curcumin (80% pure) were obtained from Sigma–Aldrich. Extract of betaine was 96% pure (Betain^{TM} 96, Trouw Nutrition, Putten, The Netherlands) and EP was a standardized extract (*E. purpurea*, 4:1, Natuur Apotheek, Pijnacker, The Netherlands). The maximum concentration of the phytochemicals tolerable to MDBK cells was determined by exposing cell monolayers to various concentrations of the compounds in cell culture medium without FCS for 20 h (37 °C, 5% CO_2) and determining the percentage of viable cells with the Trypan blue cell viability assay (Freshney, 2010). The highest concentration of each compound which allowed at least 97% MDBK viability was used in the *E. tenella* invasion assays as follows: 0.5 μg/ml betaine, 20.0 μg/ml carvacrol, 0.2 μg/ml curcumin, and 2.0 μg/ml EP. These concentrations were well below the EC50 for the phytochemicals (40, 80, 35 and 60 μg/ml respectively) estimated by plotting dose–response curves. MDBK cells were routinely maintained in media as described by Schubert et al. (2005) and were tested for contamination by plating onto blood agar plates and for Mycoplasma spp. using a kit (PlasmoTest, InvivoGen, San Diego, CA, USA).

For invasion assays, cells were seeded (3×10^4 cells/well) onto glass cover slips in 12 wells plates and incubated to 70–80% confluency in four days and the medium was replaced with DMEM containing the phytochemicals. Salinomycin (Fluka) 50 μg/ml was used as positive control (maximum inhibition); negative controls were wells containing DMEM with no additions (maximum invasion). Sporulated oocysts of *E. tenella* (Houghton strain) were supplied by Animal Health Services, Deventer, The Netherlands, prepared as described by Vervelde et al. (1998) and resuspended in HBSS pH 8.0 for immediate use. Sporozoites (2×10^5) were added to each well and cells were incubated at 37 °C in 5% CO_2. The sporozoite suspension was then discarded, cells were washed three times using DPBS, fixed and stained with hematoxylin–eosin (HE, Sigma) according to Augustine et al. (1997). Photographs were made with an Olympus DP25 camera of 10 evenly spaced fields per well using a Zeiss Axioskop light microscope (400×). Invasion was quantified by counting the number of cells and the number of cells invaded by sporozoites summed over 10 evenly spaced fields per well.

In the first experiment, cells were exposed (in duplicate) to sporozoites for periods of 2 h, 4 h and 20 h to investigate the period of time required for *E. tenella* invasion under these conditions. Combinations of the most effective inhibitors of *E. tenella* invasion, (carvacrol + EP and carvacrol + EP + curcumin) were tested in a second experiment that was carried out three times in duplicate using a 2 h exposure period. For the statistical analysis a linear mixed-effects model (Bates and Maechler, 2010) for clustered data was used with the number of invaded cells of the total cells as the binomial outcome. Experiment number was added as random effect to account for the correlated observations within experiment. In the first experiment explanatory factors were compound and time and the interaction between both. In the second experiment explanatory factor was (combination of) compound. The Aikaike's information criterion (AIC) was used for model selection. Software used for the analysis was program R version 2.11.1 (R Development Core Team, 2010).

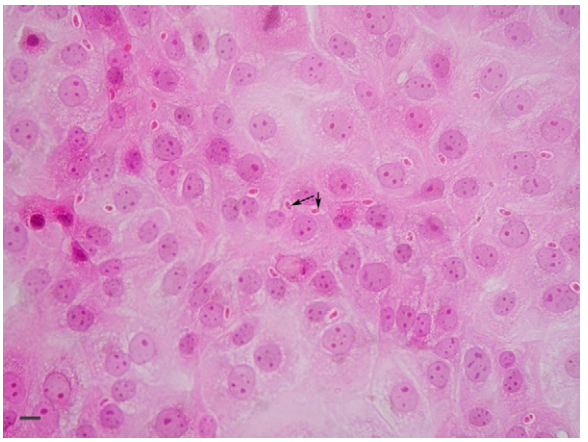

Fig. 1. MDBK cells, HE stained, showing invaded *E. tenella* sporozoites (arrow). Bar is 10 μm.

3. Results

The influence of betaine, carvacrol, curcumin and EP extract on the capacity of *E. tenella* sporozoites to invade epithelial cells was assayed by exposing a MDBK cell monolayer to sporozoites for 2, 4 or 20 h and comparing the number of cells containing one or more sporozoites after HE staining. Stained MDBK cells containing sporozoites are shown in Fig. 1.

The results for the comparison of the effect of betaine, carvacrol, curcumin and EP on the capacity of *E. tenella* sporozoites to invade epithelial cells are presented in Fig. 2 as boxplots of percentage of cells invaded. The percentage of invaded cells in the control group was about 22% (range 18–37%) whereas the betaine group had more variability and a slightly higher level of invasion. Carvacrol, curcumin and EP had a lower percentage of invaded cells with a small overlap with the control group.

After a 2 h exposure period, the odds on an invaded cell for carvacrol, curcumin and EP were significantly lower than the odds on an invaded cell in the control (i.e. lower than 1), indicating that these three compounds significantly inhibited invasion. Odds ratios (OR) were 0.71 (95%

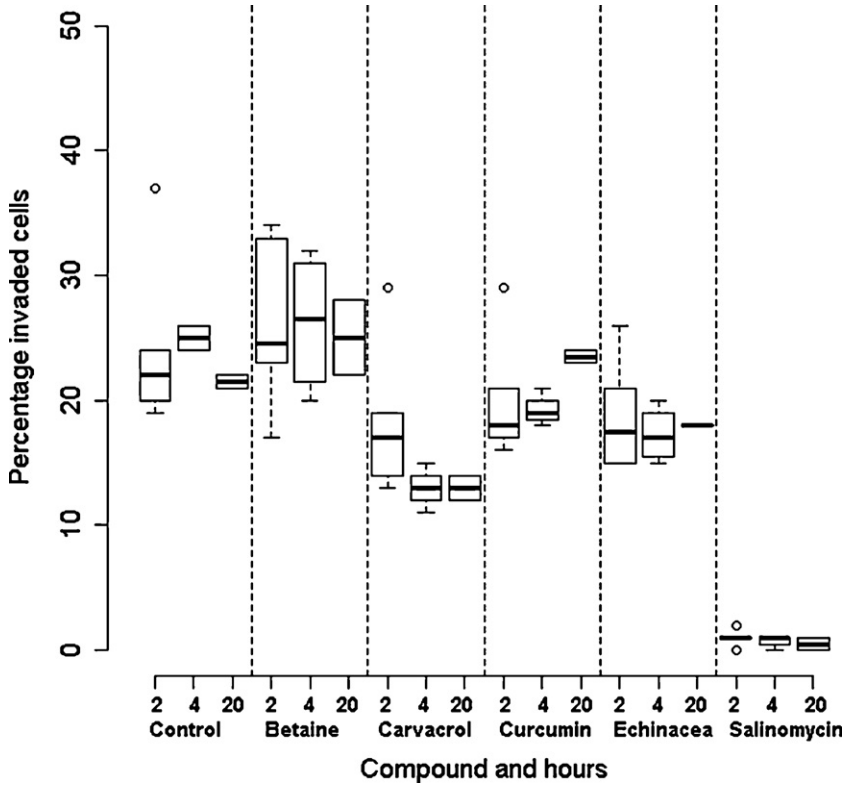

Fig. 2. Box-plot showing the effect of betaine, carvacrol, curcumin and EP on *E. tenella* sporozoite invasion into MDBK epithelial cell monolayer after 2, 4 and 20 h. Parasite invasion is expressed as a percentage of cells which contain one or more sporozoites. Control is medium only. Pos (positive control) is salinomycin. Outlying data points are shown as small circles.

CI: 0.64–0.79), 0.78 (95% CI: 0.70–0.87) and 0.73 (95% CI: 0.66–0.82) respectively. In contrast, betaine had an OR significantly greater than 1 (OR = 1.12, 95% CI: 1.02–1.24), indicating that betaine significantly enhanced invasion. As expected, the salinomycin control showed a significantly lower percentage of invaded cells compared to the untreated control (OR = 0.04, 95% CI: 0.03–0.05).

At 4 h, sporozoite invasion percentages for untreated control (OR = 1.24, 95% CI: 1.10–1.39), betaine (OR = 1.25, 95% CI: 1.09–1.36) and curcumin (OR = 1.15, 95% CI: 1.02–1.30) were significantly higher compared with the same treatment at 2 h. From 4 h to 20 h there was for the untreated control (OR = 1.00, 95% CI: 0.86–1.15) and betaine (OR = 1.08, 95% CI: 0.95–1.24) no significant change but for curcumin there was a significant increase in invasion (OR = 1.46, 95% CI: 1.27–1.68). In the presence of EP there was no significant further change in percentage invaded cells at 4 h (OR = 1.08, 95% CI: 0.95–1.23) or 20 h (OR = 1.08, 95% CI: 0.93–1.27) compared to 2 h. In contrast, the presence of carvacrol led to a significant decrease in percentage invasion at 4 h (OR = 0.78, 95% CI: 0.68–0.89) and again at 20 h (OR = 0.75, 95% CI: 0.64–0.88) compared to 2 h.

To investigate whether combining two or three compounds in the same medium could improve inhibition of *E. tenella* invasion further, combinations of the three inhibitive phytochemicals were tested as follows: carvacrol + EP, and carvacrol + EP + curcumin. MDBK cell viability was not adversely affected (>97%) in either case. The results for the phytocombinations and ethanol vehicle are presented in boxplots in Fig. 3. In the control group the percentage of invaded cells was around 20% (range 13–34%) and the salinomycin control (OR = 0.08, 95% CI: 0.06–0.10) was significantly lower at about 5% with a small variation. Carvacrol (OR = 0.69, 95% CI: 0.60–0.78) and the combinations carvacrol + EP (OR = 0.40, 95% CI: 0.36–0.45) and carvacrol + EP + curcumin (OR = 0.47, 95% CI: 0.42–0.52) had significantly lower percentages of invaded cells (range 8–18%) compared to the control. EP (OR = 0.98, 95% CI: 0.87–1.11) and ethanol (OR = 0.97, 95% CI: 0.88–1.07) were not significantly different from the untreated control.

4. Discussion

The percentage of cells invaded under control circumstances in this study was comparable with other studies using the same cell line and species of parasite (Khalafalla et al., 2011), therefore the method used may be considered an acceptable model.

To our knowledge this is the first study to evaluate carvacrol or EP as inhibitors of *E. tenella* invasion in vitro. Our findings for curcumin confirm the results of another study on the inhibitory effect of curcumin on the activity of *E. tenella* sporozoites (Khalafalla et al., 2011). Our finding that betaine enhances invasion by *E. tenella* into a cell monolayer concurs with an earlier in vitro study (Augustine et al.,

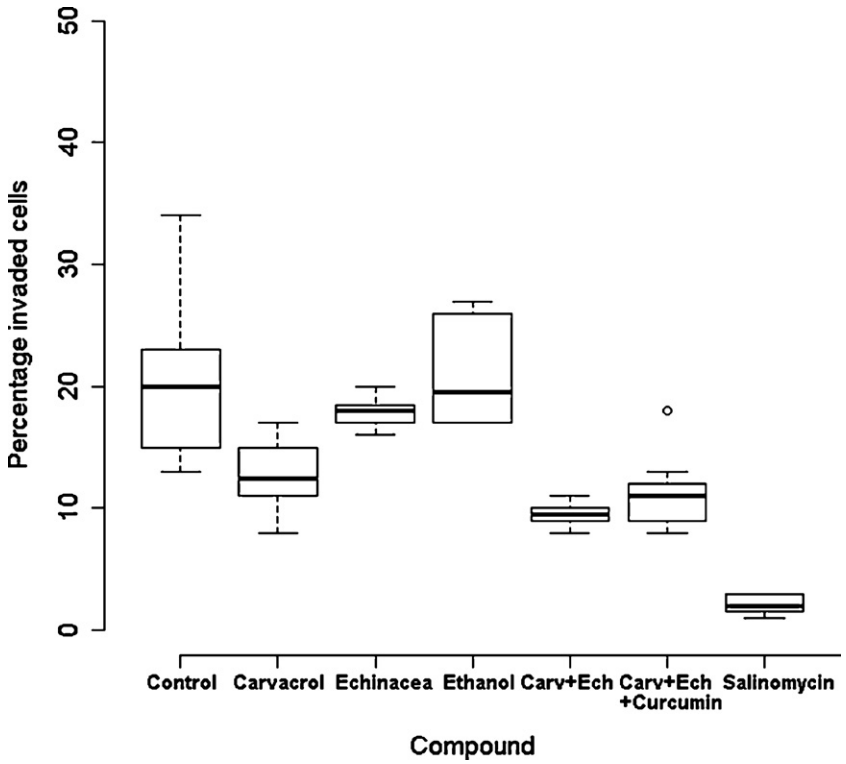

Fig. 3. Box-plot showing the effect of combinations of phytochemicals on *E. tenella* sporozoite invasion into MDBK epithelial cell monolayer after 2 h. Parasite invasion is expressed as a percentage of cells which contain one or more sporozoites. Negative controls are control (medium only) and ethanol (solvent control). Pos (positive control) is salinomycin. Outlying data points are shown as small circles.

1997). The mechanism of enhanced invasion is unknown, but may be linked to osmotic effects attributed to betaine (Health Council of the Netherlands, 2003).

Schubert et al. (2005) have demonstrated that extracellular calcium and Ca^{2+} signaling are essential for the invasion of *E. tenella* sporozoites into host cells. Carvacrol has been shown to activate and desensitize receptors in calcium channels (Sarkozi et al., 2007). It is possible that carvacrol contributes to the observed inhibition of sporozoite invasion by disrupting calcium-mediated signaling in the sporozoites.

EP is a plant extract and therefore contains several compounds, such as alkamides, polysaccharides and caffeic acid glycosides. Such preparations can exert immune stimulant or immune modulatory properties, depending on the exact composition and the pharmacology of *Echinacea* spp. extracts have been reviewed (Barnes et al., 2005).

Incubation of *E. tenella* sporozoites with curcumin can produce morphological changes and reduce sporozoite viability, but these changes are seen only after incubation for 48 h at curcumin concentrations tenfold higher than used in the present study, so it is unlikely that the invasion inhibition we have observed is due to reduced viability (Khalafalla et al., 2011). Antiparasitic activity of curcumin has been tested against *Cryptosporidium parvum*; 100–200 μM curcumin significantly reduced invasion of *C. parvum* into a HCT-8 cell monolayer (Shahiduzzaman et al., 2009). One of the possible targets for curcumin action could be a Ca^{2+}–ATPase, since curcumin can inhibit mammalian Ca^{2+}–ATPase with an IC50 of about 7–15 μM (Reddy et al., 2005).

The proportion of cells invaded by sporozoites decreased from 2 to 4 and 20 h in the presence of carvacrol but increased with time for curcumin (Fig. 2). The assay used in this study measures the net effect on sporozoite ability to invade epithelial cells within a given time period and the effect of the phytochemicals on the motility or viability of sporozoites was not investigated. It is known that *E. tenella* sporozoites can enter and leave several cells before final invasion (cell wounding) (Wiersma et al., 2004). If at the concentrations used in this study carvacrol is cytotoxic to sporozoites and curcumin is non toxic (vide supra), the longer exposure period would reduce invasion capability in the case of carvacrol but enable more sporozoites to invade in the case of curcumin.

Combining carvacrol with EP in the medium inhibited *E. tenella* invasion more effectively than applying the compounds individually, but a further addition of curcumin did not enhance performance any more (Fig. 3). Recent in vivo studies have shown that combinations of phytonutrients can have a beneficial effect on immune response and growth performance during *Eimeria* infection in poultry; the feeding of combinations of phytonutrients (including carvacrol) against *E. tenella* in chickens demonstrated enhanced coccidiosis resistance via regulation of gene expression in the chicken gut (Lillehoj et al., 2011). The combination cinnamaldehyde–carvacrol–capsaicin enhanced immune

responses to *E. tenella* vaccination in chickens (Lee et al., 2011).

The model of parasitic invasion used here to compare the performance of inhibitory phytochemicals was limited to one concentration of each phytochemical as being the maximum tolerable to the MDBK cells. The extent to which the present combinations of products have clinical relevance has not yet been determined. More research would be required to elucidate the mechanism of action and for in vivo trials. The phytochemicals studied here are not proposed as direct replacements for coccidiostats but as candidate feed additives as synergists or adjuvants in parallel to vaccination and/or coccidiostats.

In conclusion, this study shows that invasion of MDBK epithelial cells by *E. tenella* sporozoites is inhibited in the presence of carvacrol, curcumin, or EP and enhanced by betaine. The combination of carvacrol with EP inhibited sporozoite invasion further. There may be potential for developing phytochemicals as anti-coccidial feed or water additives for poultry.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was part of the project Modulation with Immune-stimulating Phytochemicals (ModIPhy) in the Immuno Valley ALTANT programme funded by The Netherlands Ministry of Agriculture, Nature and Food Quality. The sponsors had no involvement in the study design, data collection and analysis or in the writing and submission of the manuscript. The authors thank F. van Knapen and H. Peek for technical discussions and A. Timan for technical assistance. MDBK cells were a kind gift from N. Schuurman, Utrecht University.

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