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Effect of Selenium-Rich Green Tea Extract on the Course of Sporulation of Eimeria Oocysts

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Abstract: *The effect of green tea extracts on the sporulation of oocysts of 3 species of Eimeria was investigated. The unsporulated oocysts were exposed to 0%, 5% and 10% of green tea extracts (1 and 2% strengths, w/v). A new sporulation inhibition bioassay was designed to evaluate the anticocccidial activity of the tea extracts. The results showed for the first time that water-soluble extracts from green tea have anticoccidial activity as evidenced by their ability to significantly decrease (P< 0.0001) the sporulatuion of the oocysts of three species of Eimeria, namely Eimeria tenella, E. maxima and E. acervulina under laboratory conditions. Incubation of unsporulated oocysts of these parasites in water containing 10% and 25% of tea extract resulted in inhibition of sporulation of these oocysts by about 28-84% relative to the oocysts in the control incubations (no tea extract added). In addition, the sporocysts in about 5-30% of the sporulated oocysts of the three species of Eimeria recovered from incubations containing the tea extract were abnormal in their shapes and sizes. In conclusion, water-soluble extracts of selenium-rich green tea demonstrated anticocidial activity as evidenced by their ability to decrease the sporulation process and via interference with the development of the sporocysts.*
Keywords: *Selenium-rich green tea, Eimeria oocysts, sporulation assay, new assay*

I. Introduction

Globally, the consumption of poultry products increases every year and consequently the demand for poultry products by the consumers also increases yearly. This led to a rapid growth of the poultry industry via increasing the numbers of commercially held chickens which in turn increased the number of birds at risk of getting infected with various bacterial, viral, and parasitic diseases. Globally, coccidiosis is the major parasitic disease of poultry and costs the poultry industry billions of dollars every year [1, 2].

Coccidiosis is caused by many species of Eimeria which are intracellular protozoan parasites belong to the phylum Apicomplexa. At least seven Eimeria species can infect chickens and cause coccidiosis [3]. The disease transmits among the birds by the oocysts, released in the feces of the infected birds, which are resistant to environmental conditions and remain infectious for a long time.

Increasing resistance of avian coccidia to currently used anticoccidial drugs (Williams, 2006), coupled with the increasing costs of developing new drugs/vaccins and the public's distrust of drug-treated meat demonstrate the need for alternative methods of controlling this disease.

Green tea is derived from the unfermented leaves of Camellia sinensis and consequently retains the highest concentrations of polyphenols (catechins) in comparison with other teas (reference). This tea is a widely consumed beverage and has been thought to possess significant health-promoting effects such as lowering the incidence of cancer, oxidative stress-induced neurodegenerateive diseases, antioxidant, antibacterial and antiparasitic activities [4-14]. The green tea used in this study is unique as it contains high level of organic selenium in addition to the phenolic compounds and other elements [11].

This study was a part of a programme aiming to find alternative strategies [15] to treat coccidiosis in chickens using polyphenols-containing forages and medicinal plants. The purpose of this study was to evaluate the effects of green tea on the sporulation of 3 species of Eimeria (E. tenella, E. acervulina and E. maxima) under in vitro conditions.

II. Materials And Methods

2.1. Preparation of water-soluble extracts

Dried loose leaves of selenium-containing green tea (Se-BTE) were obtained from a tea plantation in China which is only 19 km away from Se mine. In addition to the phenolic compounds and other minerals, this tea contains 1.4 mg of organic selenium/kg dry leaves [11].

The water extracts for green tea were made by adding 100 ml water (100 ^{0}C) to 1 g (1% strength extracts) or 2 g (2% strength extracts) tea leaves and allowed to brew for 10 minutes with stirring. The extracts were freshly prepared, cooled and used in the assay. From each tea strength, two concentrations/dilutions (5 and 10%, v/v) were used.

2.2. Sample preparation and experimental design

A new sporulation inhibition bioassay was designed to evaluate the anticocccidial activity of the tested materials (Fig. 1). The design consists of a constant temperature water bath, silicon soft tubes, air pumps, metal valves to be inserted at one end of the silicone tube in order to control the amount of air, disposable small pipette tips to be inserted at one end of the silicone tube and then inside the experimental tube for bubbling, and a piece of wood on which the metal valves can be attached.

The unsporulated oocysts of pure species were obtained from Avivet Ltd, Palmerston North, New Zealand. The oocysts were used within 2-4 hours of collection and the numbers of oocysts were assessed using a McMaster counting chamber [16]. For each species of Eimeria, fifteen 5-ml plastic tubes, three for each treatment, were labeled and prepared for incubation, and 0.5-ml aliquots of oocyst solution were withdrawn and randomly allocated to each tube into which various concentrations of tea extracts were added. The oocyst solution was thoroughly mixed using magnetic stirrer bar/plate at a low speed.

In this bioassay (Fig. 1), the unsporulated oocysts were incubated with tea extracts for 48-72 hours, depending on the species of Eimeria, at 27-29 ^{o}C using digital water bath to maintain constant temperature. The oocysts were gently aerated with an air pump away from sun light (Fig. 1). At the end of the incubation, the numbers of sporulated and non-sporulated oocysts were counted and the sporulation percentage was estimated by counting the number of sporulated oocysts in a total of 100 oocysts. In addition, the numbers of sporocysts within each sporulated oocyst were counted and the numbers of abnormal sporocysts were counted.

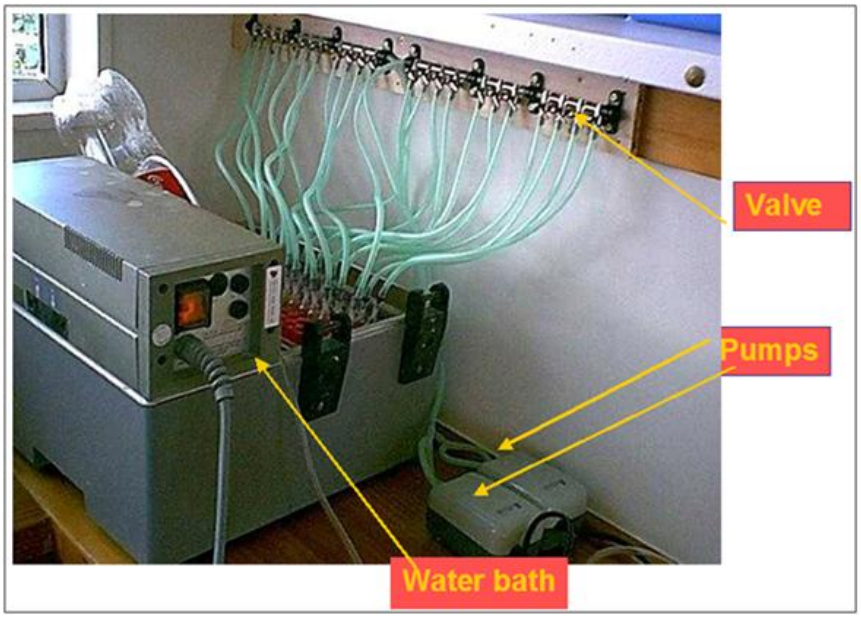

Fig. 1. Newly established bioassay for determining the sporulation rate of coccidian oocysts.

2.3. Statistical analysis

One way ANOVA was carried out and the differences between the groups were tested with Duncan's test using a statistical package program (SPSS version 18.0). A difference was considered to be statistically significant when $p < 0.05$.

III. Results

The percentage sporulation (upper panel) and % inhibition of sporulation (lower panel) of Eimeria tenella oocysts are presented in Fig. 2. About 81% of the oocysts of E. tenella managed to sporulate in the control incubations containing oocysts and tap water only (Fig. 2, upper panel). In contrast, in incubations containing 5% (v/v) and 10% (v/v) of 1% strength (w/v) green tea extract only 47.5% and 29% were able to sporulate, respectively ($P< 0.0001$; relative to control incubation). Results shown in Fig. 2/lower panel revealed that green tea treatment at concentrations of 5% and 10% resulted in 41.4% and 64.2% reduction in sporulation, respectively relative to the control incubation.

In incubations containing 5% and 10% of 2% strength green tea extracts only 41% and 14% of the oocysts were able to sporulate, respectively ($P< 0.0001$; relative to control incubation) and this corresponds to 49.4% and 82.7% reduction in sporulation process (Fig. 2, lower panel), respectively.

It is important to mention that about 5-20% of the sporulated oocysts that exposed to green tea were containing abnormal sporocysts (data not included) as evidenced by their abnormal sizes and shapes (Fig. 3B-D) in comparison to those obtained from control incubations (no tea added, Fig. 3A).

In control incubations containing water and E. acervulina unsporulated oocysts, 84.5% of these oocysts were able to sporulate (Fig. 4, upper panel). In incubations containing 5% and 10% of green tea water-soluble extract (1% strength), 23.1% and 51.5% were not able to sporulate, respectively (Fig. 4, lower panel) whereas in incubations containing 5% and 10% of 2% strength green tea extract, 28.4% and 84.02% of the oocysts were not able to sporulate, respectively (P< 0.0001; relative to control incubation). It is important to mention that less than 5% of the sporulated oocysts were containing sporocysts of abnormal shapes and sizes (data not shown).

The efficacy of same tea extracts were tested against the oocysts of E. maxima and the results are presented in Fig. 5. About 88.5% of the oocysts in the control incubations were managed to attain full sporulation (Fig. 5/upper panel). In incubations containing 5% and 10% of 1% strength green tea water-soluble extract, 71% and 44% were able to sporulate, respectively whereas in incubations containing 5% and 10% of 2% strength green tea extract, 63.5% and 30% of the oocysts were able to sporulate, respectively (P< 0.0001; relative to control incubation). Results shown in Fig. 5 (lower panel) revealed that green tea treatment at concentrations of 5% and 10% resulted in 19.8% and 50.3% reduction in sporulation, respectively when the tea strength was 1% whereas they resulted in 28.3% and 66.14% reduction, respectively at the strength 2% relative to the control incubation.

About 8-30% of the oocysts (data not shown) exposed to tea extracts were containing sporocysts with abnormal shapes and sizes and some are shown in Fig. 6. A slightly flattened sporulated oocyst of E. maxima containing 4 typical sporocysts is shown in Fig. 6A. By comparison, the oocysts exposed to tea extracts showed a large proportion of divided but degenerate forms with the sporont contorted into bizarre shapes, indicating abortive attempts at division (Fig. 6B-D).

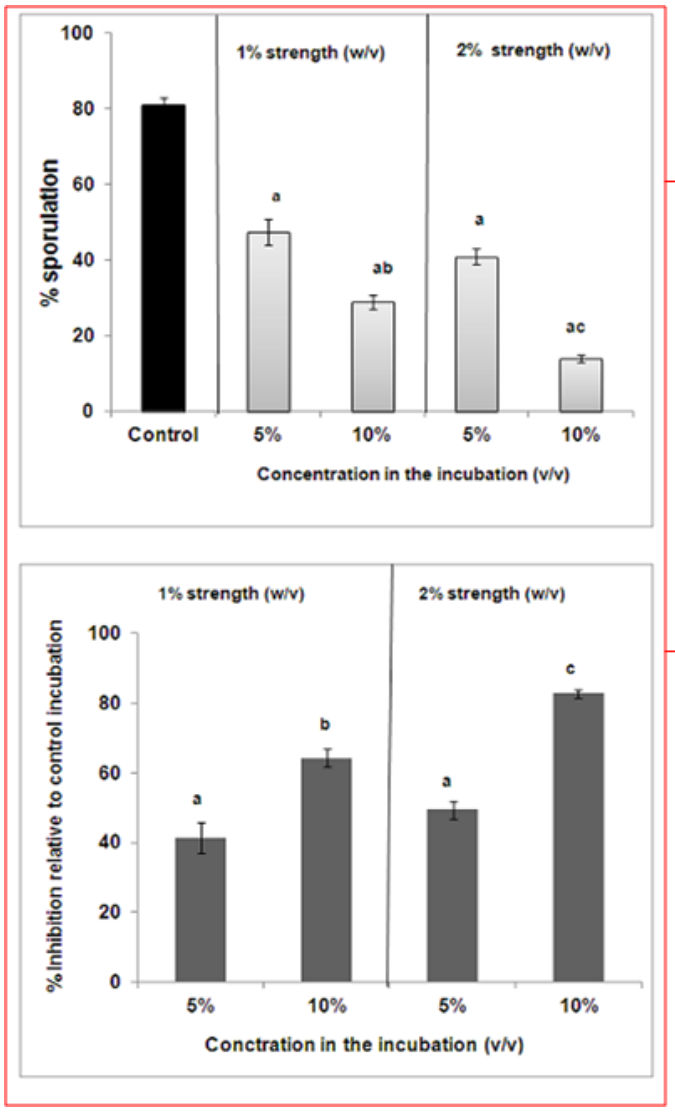

Fig. 2. Percentage sporulation (upper panel) and percentage inhibition of sporulation (lower panel) of the oocysts of *Eimeria tenella* oocysts in incubations without (control) and with selenium-rich green tea under *in vitro* conditions. Values are means ± SE. **a** significant against control incubations (*P<0.0001*); **b** and **c** significant against incubations containing different concentrations of tea extract (*P<0.0001*).

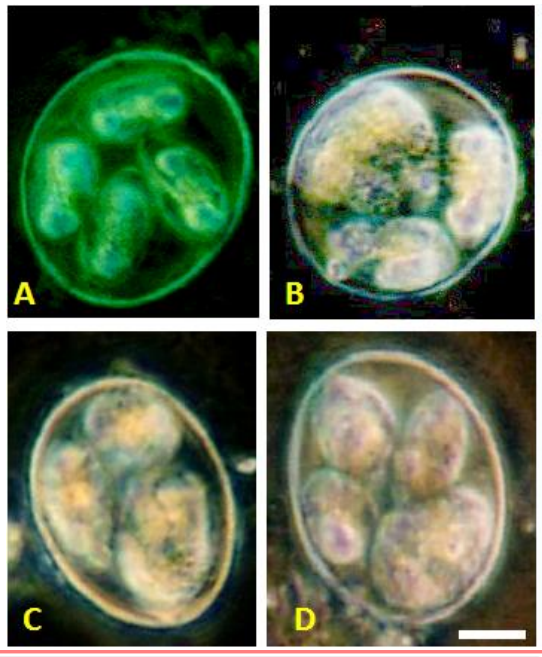

Fig. 3. Photo-micrographs of oocysts of *Eimeria tenella*. **A**: sporulated oocyst from control incubation (no tea extract added) with 4 typical normal sporocysts. **B, C** and **D** are oocysts containing abnormal sporocysts (note the size and shape of the sporocysts inside the oocysts) recovered from incubations containing different concentrations of tea extract. Oocysts were incubated for 48 hours in the presence or absence of tea extracts and were viewed under bright field of microscopy (1000x total magnification). Exposure to tea extracts caused changes in sporocyst numbers and morphology. Scale bar (A-D) = 20 µm.

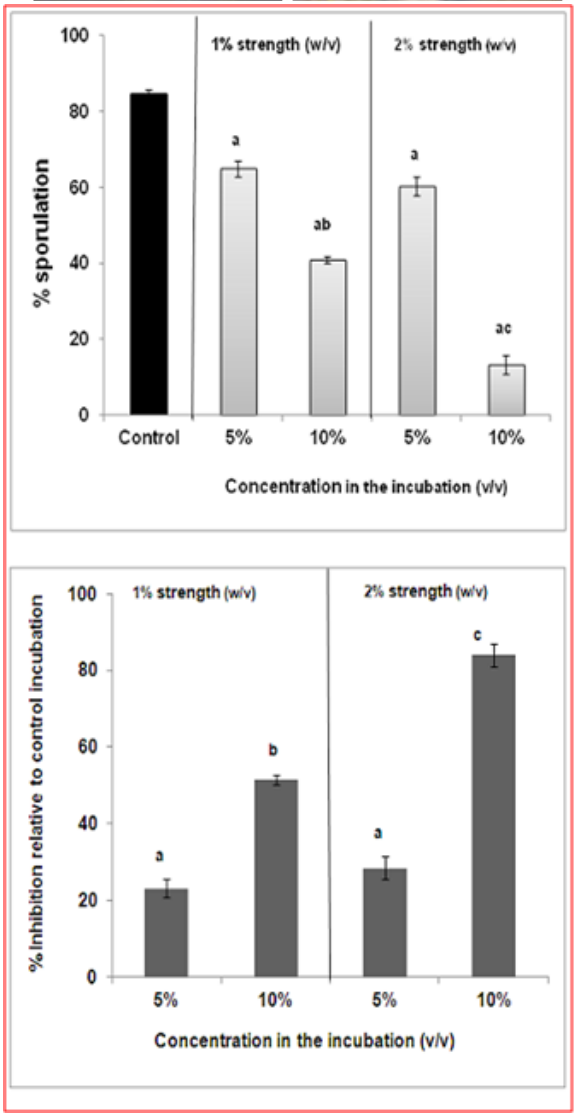

Fig. 4. Percentage sporulation (upper panel) and percentage inhibition of sporulation (lower panel) of the oocysts of *Eimeria acervulina* oocysts in incubations without (control) and with tea extract under *in vitro* conditions. Values are means ± SE. **a** Significant against control incubations (*P<0.0001*); **b** and **c** significant against incubations containing different concentrations of tea extract (*P<0.0001*).

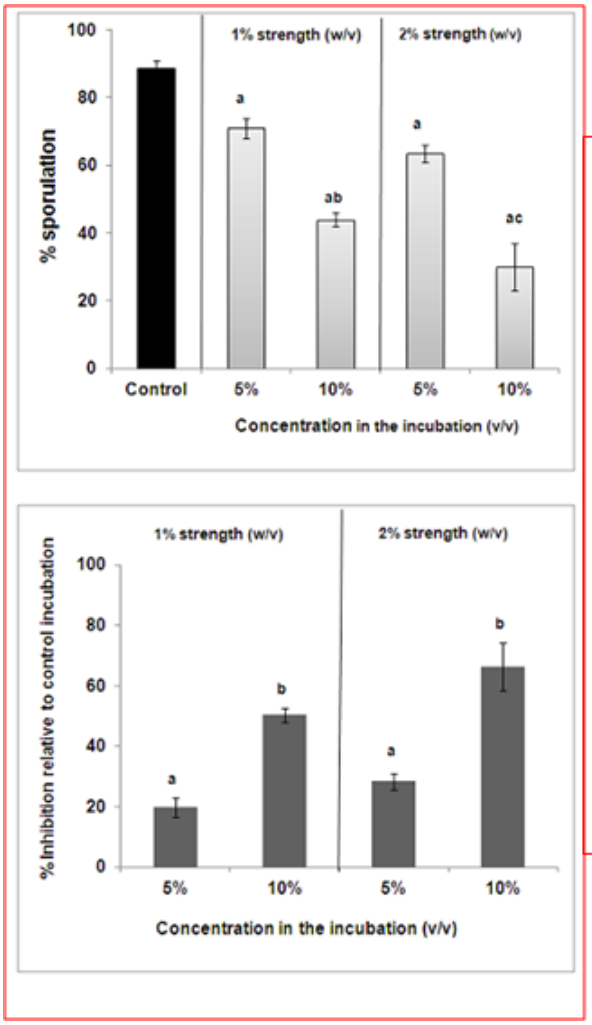

Fig. 5. Percentage sporulation (upper panel) and percentage inhibition of sporulation (lower panel) of the oocysts of *Eimeria maxima* oocysts in incubations without (control) and with tea extract under *in vitro* conditions. Values are means ± SE. **a** significant against control incubations (*P<0.0001*); **b** and **c** significant against incubations containing different concentrations of tea extract (*P<0.0001*).

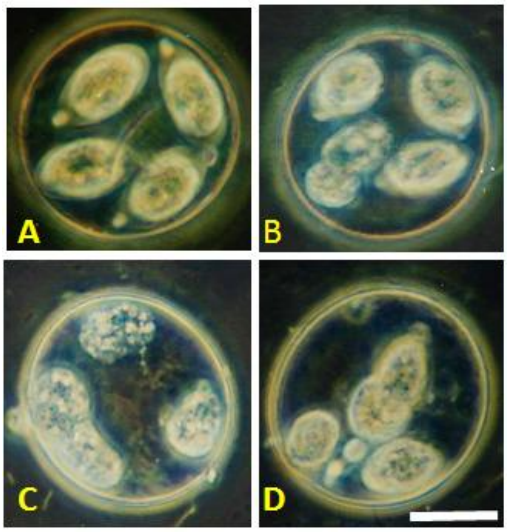

Fig. 6. Photo-micrographs of oocysts of *Eimeria maxima*. **A**: sporulated oocyst from control incubation contains 4 typical normal sporocysts. **B, C** and **D** are oocysts with abnormal sporocysts recovered from incubations containing different concentrations of tea extract. Oocysts were incubated for 48 hours in the presence or absence of tea extracts and were viewed under bright field of microscopy (1000x total magnification). Exposure to tea extracts caused changes in sporocyst numbers and morphology. Scale bar (A-D) = 20 µm.

IV. Discussion

The results of the present study show for the first time that water-soluble extracts from selenium (se)-rich green tea have anticoccidial capacity as evidenced by the ability to decrease the sporulation process of the oocysts of three Eimeria species, namely E. tenella, E. acervulina and E. maxima, under laboratory conditions. Based on the fact that the number of coccidian sporulated oocysts and course of sporulation is an important factor in terms of the level of challenge to which chickens are exposed, the results of the present study are very important from the epidemiology point of view. However, the difficulty of controlling the contamination of

poultry houses with coccidian oocysts is very well documented [17] and consequently the negative effects of coccidian infection on technical and economic results persist [18].

If the oocysts recovered from chickens fed diets supplemented with green tea fail to sporulate, this means that the buildup of infective oocysts in the poultry houses litters will be lessened, thus markedly reducing the potential for future infections. This consequently will reduce the usage of anticoccidial drugs which will reduce the dependence on anticoccidial drugs as the sole method of controlling coccidia in poultry and reduce the possibility of developing drug resistance. This effect will be more likely to happen under in vivo conditions based on the fact that a high percentage of polyphenols and tannins is not absorbed from the gastrointestinal tract and leave the body with feces [19-21] and consequently the oocysts shed from the infected birds will be exposed to the inhibitory effect of polyphenolic compounds throughout their sporulation period.

Hur et al. [21] studied the effects of condensed tannin (CT)-containing plants on the coccidian infection in goats and found that feeding fresh pine needles and dry oak leaves in combination with lucerne chaff had anticoccidial activities in goats as demonstrated by a sharp decrease in the numbers of oocysts shed by these goats in comparison with the control goats fed lucerne chaff only. Jang et al. [9] studied the anticoccidial effects of green tea-based diets in chickens following oral infection with Eimeria maxima and found that the green tea-fed chicken produced significantly reduced fecal oocysts when compared to the E. maxima-infected group fed standard diet but it seems that the authors didn't check the sporulation rate of the shed oocysts. Sannella et al. [22] investigated the antimalarial properties of green tea and they found that crude extract of green tea and two of its constituents, epigallocatechin-3-gallate and epicatechin gallate, strongly inhibit Plasmodium falciparum growth in vitro. Recently, Molan et al. [15] studied the impact of water extracts from pine (Pinus radiata) bark on the sporulation of three species of avian coccidia and found that these extracts have the ability to decrease significantly the sporulation of the oocysts of these species under laboratory conditions.

Although the mechanism by which the tea extracts inhibited the sporulation process is not known, the ability of polyphenolic compounds to inhibit the activities of various endogenous enzymes [23, 24] is well documented. This encouraged us to speculate that the tea extracts, which contain abundant amounts of polyphenolic compounds and selenium [10, 11], may have the ability to inhibit the enzymes responsible for the sporulation process of the coccidian oocysts.

The present study showed that E. maxima oocysts were significantly more resistant to the inhibitory action of green tea extract than the oocysts of E. tenella and E. acervulina. Similarly, Molan et al. [15] reported that the oocysts of E. maxima were more resistant to the action of pine bark extracts than the oocysts of E. tenella and E. acervulina.

The other important and novel finding of the present study is the presence of sporocysts with abnormal shapes and sizes inside the oocysts that exposed to tea extracts which may indicate the capacity of certain polyphenolic compounds in the tea extracts to penetrate the tough wall of the oocysts and interfere with the development of the sporocysts and sporozoites. Although it is difficult to compare between the bacterial cell wall and the oocyst wall, Jones et al. [25] suggested that condensed tannins (CT) may penetrate the cell wall of bacteria and cause a loss of intracellular components. In the present study, the polyphenols of green tea may penetrate the wall of the oocyst and damage the cytoplasm (sporont) as evidenced by the appearance of abnormal sporocysts in oocysts exposed to tea extract but not in those recovered from the control incubations. Similarly, Molan et al. [15] came across abnormal sporocysts in E. maxima oocysts that have been exposed to high concentrations of pine bark extracts containing 35% condensed tannins.

In order to determine whether the same effect could be achieved in vivo, an experiment is underway to evaluate the viability and infectivity of green tea-treated sporulated coccidian oocysts.

V. Conclusion

We conclude that water-soluble extracts of green tea can reduce significantly the sporulation of the oocysts of three eimerian species and modify the morphology of the sporocysts within the oocysts under in vitro conditions. This may be explained by the ability of the polyphenols in the green tea to penetrate the oocysts wall and reach the sporont and modify the trend of cellular division.

Acknowledgements

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