

The value of plant extracts with antioxidant activity in attenuating coccidiosis in broiler chickens

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Abstract

Coccidiosis remains one of the most important diseases in the poultry industry and results in the annual loss of millions of US dollars by the poultry industry. In South Africa and other developing countries where a large percentage of the population is unemployed, cheap food production is necessary. If the control of the coccidian parasite could be made more economical, these savings could be passed on to the consumer. In Europe, where the economics are different, people are becoming more aware of the potential dangers of using antimicrobials in producing animal protein. A solution to both these problems could be the use of plant products that function by mechanisms other than those of chemotherapeutics, with the additional advantage of a natural origin. Antioxidant compounds could hold promise for the control of *Eimeria* infections due to the association of coccidial infection with lipid peroxidation of the intestinal mucosa. Four plant extracts with antioxidant activity were screened for their anticoccidial activity *in vivo* with toltrazuril as the positive control. *Combretum woodii* (160 mg/kg) proved to be extremely toxic to the birds, while treatment with *Tulbaghia violacea* (35 mg/kg), *Vitis vinifera* (75 mg/kg) and *Artemisia afra* (150 mg/kg) resulted in feed conversion ratios similar to toltrazuril, and higher than the untreated control. *T. violacea* also significantly decreased the oocyst production in the birds. From this study we conclude that antioxidant-rich plant extracts have potential benefits in treating coccidial infections. The promising results obtained with *T. violacea* justify further studies on the potential value of the plant as a therapeutic or prophylactic anticoccidial agent.

Keywords: Anticoccidial; Poultry; Herbal remedies; Ethnoveterinary medicines; *Artemisia afra*; Grape seed extract; *Tulbaghia violacea*; *Combretum woodii*

1. Introduction

The South African poultry industry is the largest contributor to the agricultural economy with approximately 12 million broiler chickens being slaughtered per week (Germishuis, 2006). In a country with high unemployment rates, this industry is a very important supplier of relatively low cost protein to all sectors of the South African economy (Statistics SA, 2006). One of the major problems facing the commercial poultry industry is coccidiosis (unpublished data obtained from the South African Poultry Industry).

Although the actual losses incurred in South Africa remain unknown, they are believed to run into millions of dollars due to resultant production losses. In the United Kingdom, to which South Africa compares favourably in terms of production capacity and systems, the total cost

of coccidiosis in chickens was estimated at US \$77 million in 1995 and resulted from a combination of in-food medication costs, veterinary costs and production losses (Williams, 1999). At present South African commercial poultry farmers spend approximately US \$0.02 per bird (US \$12.5 million annually) on the use of in-feed prophylactic anticoccidials in order to limit mortalities and enhance broiler growth and production. The anticoccidials in use include the polyether (ionophor) group of chemotherapeutics, sulphonamides, pyrimidine derivatives, triazinetriones and the benzenacetone nitriles (Carrington et al., 2007).

Unfortunately, with the widespread use of anticoccidial drugs, resistance has developed to all the drugs introduced thus far ([Chapman, 1997] and [Chapman, 1998]). An additional constraint in their use comes from the consumer and the ever-increasing need for the drug-free production of foods (Harper and Makatouni, 2002). The use of plant extracts as medicants may alleviate these difficulties, as they are not only natural products but may comprise new therapeutic molecules to which resistance has not yet developed. The use of herbal remedies in the management of coccidiosis is not a new concept. For example, halofuginone, a quinazolinone alkaloid derived from *Dichroa febrifuga*, has been used as a coccidiostat, and the original extract from *D. febrifuga*, known as febrifugine, possesses antimalarial and anticoccidial activity (Youn and Noh, 2001). The investigation of herbal materials as anticoccidial remedies therefore holds promise as an alternate in the control of coccidiosis. In addition to possibly lowering the cost of food production in South Africa, it could create a herbal remedy export market and thereby create more jobs in the country. The use of herbal extracts may also satisfy the increasing concerns of consumers, on condition that they prove to be both safe and effective. South Africa possesses an enormous diversity of plant resources, which has yielded many examples of useful bioactive compounds ([Van Wyk, 2002] and [Fennell et al., 2004]). We discuss the *in vivo* effect of four herbal extracts prepared from South African plants on artificially induced coccidial infection in 2-week-old broiler chickens.

For this study *Combretum woodii* Dummer (Combretaceae), *Vitis vinifera* L. (Ampelidaceae), *Artemisia afra* Jacq. ex Willd. (Asteraceae) and *Tulbaghia violacea* Harv. (Liliaceae) were selected on the basis of previously reported antioxidant activity ([Allen et al., 1998], [Burits et al., 2001], [Kubec et al., 2002], [Zishiri, 2004], [Chikoto and Eloff, 2005], [Zheng and Wang, 2001] and [Velíšek et al., 2006]). According to Allen et al. (1998) antioxidant compounds are known to reduce the severity of *Eimeria tenella* infections by ameliorating the degree of intestinal lipid peroxidation. One of the most potent veterinary anticoccidials, toltrazuril, is believed to achieve some, if not all, of its beneficial effect by limiting the degree of lipid peroxidation (Eraslan et al., 2004).

2. Materials and methods

2.1. Plant collection and preparation

A. afra, *C. woodii* and *T. violacea* were collected in Pretoria, South Africa, in September 2005. Voucher specimens were deposited in the Herbarium of the Department of Paraclinical Sciences (Medicinal Plant Collection) for *A. afra* (voucher number McGaw200502), *C. woodii* (voucher number McGaw200503) and *T. violacea* (voucher number McGaw200504), respectively. The leaves and twigs of *A. afra*, leaves of *C. woodii*, and roughly chopped whole plants of *T. violacea* were dried at 40 °C in a forced stream of air using a custom-built plant

drying machine. The plant material was ground to a powder using a Macsalab Model 200 LAB grinder. Extracts were prepared by maceration with shaking (Labotec Model 20.2 shaker) for 24 h in 70% acetone with a 10:1 solvent to dry weight ratio (Eloff, 1998). The extracts were filtered through Whatman No 1 filter paper using a Büchner funnel, and the acetone was removed using a rotary evaporator (Büchi Rotavapor R-200). An aliquot of the remaining aqueous plant extract for each species was freeze-dried (Specht Scientific freeze-drier) to determine the concentration of the plant extracts prior to use in the anticoccidial assay.

V. vinifera (GS) extract was prepared from seeds according to a patented method developed to maximise antioxidant capacity (Chikoto and Eloff, 2005). Briefly, oil-expressed grape seed (a waste product from grape seed oil production) was washed with water and the marc extracted with 80% ethanol. The extract was filtered and passed through Diaion HP20 and dried to obtain an antioxidant-rich extract. In preparation for the anticoccidial assay, the grape seed extract powder was dissolved in a small volume of 60% ethanol and the volume subsequently increased with distilled water so that the final concentration of ethanol was 12%. The concentration represented the point of maximum solubility of the extract in water.

To assess the stability of the extracts, aliquots (100 µg) of the extracts were separated using thin layer chromatography (TLC, Merck aluminium-backed plates, silica gel 60 F₂₅₄). Three solvent systems of differing polarities were used (Kotzé and Eloff, 2002), namely ethyl acetate:methanol:water (40:5.4:5, EMW, polar, neutral), chloroform:ethyl acetate:formic acid (5:4:1, CEF, intermediate polarity, acidic) and benzene:ethanol:ammonium hydroxide (90:10:1, BEA, non-polar, basic). The extracts were analysed using TLC immediately after preparation, and again before use in the anticoccidial screening. In the interim, the extracts were stored in the dark in a cold room at 4 °C. The TLC fingerprints before and after storage were compared to assess potential degradation or other changes in chemical composition of the extracts.

To confirm the *in vitro* antioxidant scavenging capacity of the selected plants in comparison to toltrazuril, the trolox equivalent antioxidant assay was utilised. For this assay the method of Re et al. (1999) which makes use of a pre-generated radical monocation, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS⁺) (Sigma–Aldrich, SA) and trolox (water-soluble vitamin E analogue) (Sigma–Aldrich, SA) was adapted (Naidoo et al., 2006). The blue/green chromophore ABTS⁺ was produced through the reaction between ABTS and potassium sulphate (Sigma–Aldrich, SA). In this assay the antioxidant activity of a molecule/extract is related to that of trolox, by means of trolox equivalent antioxidant capacity (TEAC) values.

2.2. Animals and management

Broiler chickens were reared as a single group from day old to 2 weeks on wire mesh. The animals (492 ± 20 g) were subsequently randomly divided by tag-number into treatment groups, viz. herbal extracts (*n* = 15) per group, toltrazuril (Rx, *n* = 15), a non-treated infected (I, *n* = 20) and non-treated non-infected group (C, *n* = 20) with each group being allocated to two large cages with a single tray per group to catch faecal material. The birds had free access to water and a commercial broiler feed formulated without any anticoccidial medication ([Youn and Noh, 2001], [Christaki et al., 2004] and [Du and Hu, 2004]). The birds were housed in a closed house under negative pressure ventilation with an artificial light source. Feed intake and weight gain for each group was determined weekly from 14 days until the day of infection and subsequently every alternate day as far as practical.

The birds were infected with 0.2 ml of a mixed suspension of *Eimeria tenella* (80%), *E. maxima* (9%) and *E. avervulina* (11%), containing 500 000 viable oocysts per milliliter. The chickens were monitored daily for signs of infection, which was defined as the presence of oocysts in the faeces with concurrent bloody diarrhoea (Holdsworth et al., 2004). At the first signs of infection, chickens were treated with one of the herbal extracts, once a day for 5 days at 1 ml/bird by gavage. A treatment period of 5 days was selected as this was the estimated period of oxidant insult induced by the coccidian parasite (Koinarski et al., 2005) The negative control birds received no treatment while the positive control birds were treated with toltrazuril at the recommended dose of 20 mg/kg *per os* for 2 days.

2.3. Observations and analytical procedures

All statistical analysis was undertaken using the programme SPSS 13 (SPSS Inc.). Pooled oocyst counts, per group, were undertaken as previously described by Holdsworth et al. (2004). Individual oocyst counts were not feasible due to the inability to house birds individually. In total 10 g of faeces was collected and processed. The changes in oocysts per gram (OPG) over time were evaluated using the paired sample *t*-test.

The mean weight gain (MWG) was calculated using the formula: $MWG = (\text{mean final weight of live birds in a cage or pen}) - ((\text{mean initial weight of all birds in that cage or pen}) + (\text{weight of dead birds}))$. Differences in the average weights per group for each time point were compared using ANOVA to determine if a significant difference was present between the treatment groups. Weight gains for each group for the different time points were compared using a paired sample *t*-test to ascertain if weights differed significantly over the growth period.

The group feed conversion ratio (FCR) for the study period was calculated using the formula: $FCR = \text{feed consumed per group (g)} / (\text{weight gain of surviving birds} + \text{weight gain of dead birds})$. Individual FCR were not calculated as animals were fed as a group. Changes in FCR over time were evaluated using the paired *t*-test.

The mortality rate was determined using the formula: $\text{percent mortality} = (\text{total number of dead chicks in the cage} / \text{initial number of birds in cage}) \times 100$ and evaluated for death rate and hazard analysis after being exposed to the infectious agent in the absence and presence of the herbal extract. Necropsies were performed on all mortalities. Five birds from the control group were euthanised at the first signs of infection by carbon dioxide inhalation. Full post-mortem examinations and histopathological scoring of lesions was done. Five birds were subsequently euthanised on day 32 and the remaining birds at 39 days of age. After slaughter the intestinal tract was examined and scored using a score of 0–4 (Johnson and Reid, 1970).

3. Results

All the plant extracts showed adequate antioxidant activity, with toltrazuril being more effective than the plant extracts (TEAC = 1.6). TEAC values in decreasing order were 1.2, 0.75, 0.5 and 0.2 for Grape seed extract, *A. afra*, *C. woodii* and *T. violacea*, respectively.

The final concentrations of plant extract administered to the chickens were 210 mg/ml (160 mg/kg) (*C. woodii*), 200 mg/ml (75 mg/kg) (GS), 100 mg/ml (150 mg/kg) (*A. afra*) and 51 mg/ml (35 mg/kg) (*T. violacea*). These were designated as the maximum concentration of prepared extract soluble in water. TLC analysis revealed no noteworthy changes in the chemical composition of the extracts during storage at 4 °C until administration to the chickens.

Once infected it took approximately 5 days for the animals to demonstrate clinical signs of infection, which was mild. The necropsy confirmed the low virulence of infectivity with only a few of the infected animals displaying macroscopically visible lesions. Microscopically the lesions varied in their severity with no consistency between any of the treated animals.

When comparing the weight gains between the various times, the animals treated with *C. woodii* were the most severely affected, with the MWG (Fig. 1) differing significantly to all the other groups from the start of clinical signs of infection to euthanasia. No differences were present among *A. afra*, GS, *T. violacea* and Rx from the time of clinical signs to euthanasia even though they all differed significantly from C and I. At the termination of the study (39 days) the average weight of a chicken per group was 1.8, 2.1, 2.0, 2.0, 2.0, 2.1 and 2.7 for *C. woodii*, *A. afra*, GS, *T. violacea*, Rx, I and C groups, respectively.

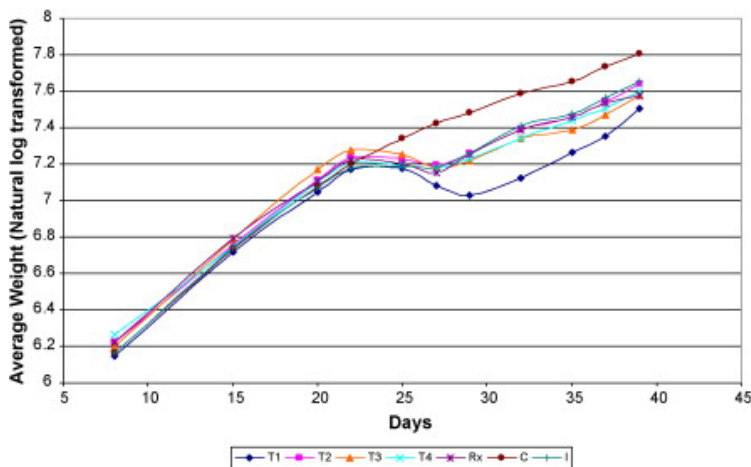


Fig. 1. Average weight gains per treatment group over time. T1: *C. woodii*; T2: *A. afra*; T3: Grape seed; T4: *T. violacea*; I: infected non-treated; Rx: toltrazuril, C: non-treated non-infected. Birds were infected on day 21, treated on day 27–31.

When comparing the FCR between the groups the animals in the control group had a ratio of 1.94 for the study. The FCR was poorer for all infected animals from the time of infection to recovery, i.e. all birds lost weight while infected. The *C. woodii* group had the poorest FCR of 6.6 which remained poor until euthanasia. When comparing the other treatment groups to the FCR of 2.9 for I, GS and Rx had a similar FCR of 2.46, which was marginally poorer than 2.36

for *A. afra* and 2.43 for *T. violacea*. Although the FCRs in general appear poor, this was influenced by the greater wasting by the birds being in cages and not on the floor.

From the OPG of faeces (Fig. 2), Rx decreased the OPG after the recommended duration of treatment and was superior to the effects of the plant extracts. Once treatment was stopped, the OPG increased in the treatment group transiently before decreasing at the same rate as the control. For the plant extracts, only *T. violacea* differed significantly from I. In addition to decreasing the OPG moderately, the lower levels were maintained for the duration of treatment, and increased on cessation of therapy.

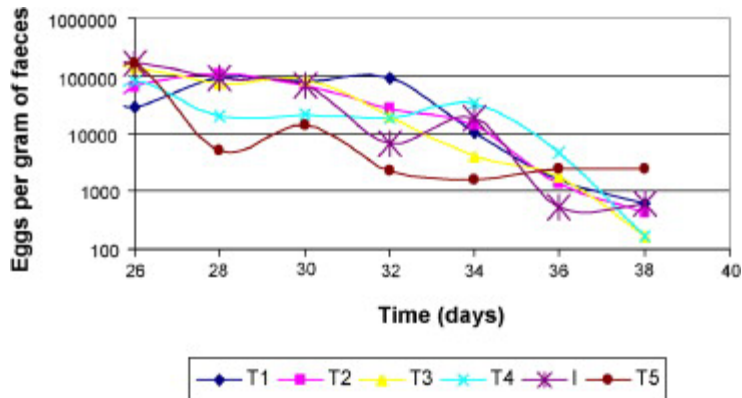


Fig. 2. Oocyst production over time for each of the treatment groups (natural logarithm transformed). T1: *C. woodii*; T2: *A. afra*; T3: grape seed; T4: *T. violacea*; I: infected non-treated; Rx: toltrazuril. Birds were infected on day 21, treated on day 27–31.

Mortalities were 6 and 1 for *C. woodii* and I, respectively with the mean survival time being 2 days shorter for animals receiving *C. woodii* compared to the other groups. *C. woodii*-treated birds also had the greatest hazard of dying. No mortalities were seen in the other groups.

4. Discussion

In all cases, the herbal extracts produced an improved FCR over the control in a similar manner to that for toltrazuril. In addition, *T. violacea* also marginally reduced oocyst shedding in the infected birds. This therefore supports previous findings on the importance of antioxidant compounds in the management of coccidiosis (Allen et al., 1998). With the coccidian parasite-induced host cell destruction being associated with oxidative stress and lipid peroxidation, the antioxidant which have the ability to neutralise reactive oxygen species, are protective due to their ROS scavenging ability. In the case of *T. violacea*, the antioxidant compounds have been identified as S-(methylthiomethyl)cysteine sulfoxide (marasmine), bis[(methylthio)methyl] disulfide and various derivatives ([Zheng and Wang, 2001], [Dictionary of Natural Products, 2006] and [Velíšek et al., 2006]). Grape seeds contain many compounds with antioxidant activity, including monomeric flavanols (catechin and epicatechin), dimeric,

trimeric and polymeric procyanidins, and phenolic acids (gallic acid and ellagic acid) ([Yilmaz and Toledo, 2004] and [Naidoo et al., 2006]).

The inability of the other plant extracts to reduce the OPG as described for toltrazuril is not known at this stage. Biophasic availability may be the problem as the extracts were administered in water firstly as it is a non-toxic solvent, and secondly to minimise systemic exposure, i.e. lower the bioavailability. With the coccidial parasite being intracellular, a degree of lipid solubility would appear to be important. Toltrazuril is very lipid soluble and is likely to achieve a higher intracellular concentration than water-soluble compounds such as catechin in grape seed. It should also be noted that marasmin, the main antioxidant in *T. violacea*, is also lipid soluble.

Irrespective of the treatments, with the exception of *C. woodii*, the birds recovered at the same rate. The natural recovery of the birds was expected as no re-infection could occur with birds not being in direct contact with their faeces. It was interesting to note that irrespective of treatment, all birds stopped shedding oocysts at the same point, suggesting that this was related more to the life cycle of the parasite rather than to the treatment.

With the poor growth and increased mortality caused by *C. woodii* we suspect plant toxicity. At present no specified toxicant has been reported to occur in the plant. This therefore appears to be the first case of *Combretum* toxicity that has been reported in birds.

5. Conclusions

With the three plant extracts promoting a similar FCR to toltrazuril, the use of antioxidant-containing plants may be of benefit in the management of coccidiosis. It is important to note that the active plant constituents should be sufficiently lipid soluble to penetrate intracellularly to have an effect on the coccidian parasite. *T. violacea* displayed a beneficial effect on the rate of oocyst shedding, showing the most promise of all the plant extracts tested in this study. This provides a strong rationale for further evaluation of the anticoccidial efficacy of *Tulbaghia violacea* plant extracts in a larger study. This plant may also have merit in the prophylactic management of coccidiosis.

Acknowledgements

The Claude Leon Foundation and the University of Pretoria are thanked for financial support (LJM). The project was funded by the National Research Foundation of South Africa (NRF).

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