Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2022.082

RESEARCH ARTICLE

Anticoccidial Efficacy of *Citrus sinensis* Essential Oil in Broiler Chicken

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ARTICLE HISTORY (22-352)

Received:	October 13, 2022
Revised:	November 09, 2022
Accepted:	November 14, 2022
Published online:	November 16, 2022

Key words:
Anticoccidial
Citrus sinensis
Coccidiosis
Essential oil
In-vivo

ABSTRACT

Due to the emergence of drug resistance against synthetic chemicals, effective alternatives like essential oils are required for the control of coccidiosis in poultry. Hence, the present *in-vivo* experiment was conducted on *Citrus (C.) sinensis* essential oil for investigation of its anticoccidial efficacy. For this purpose, 72 day-old chicks were procured from local market and divided randomly into six equal groups**:** A, B, C, D, E and F. On day 14, all the groups except F were administered with 50,000 sporulated oocysts (mixed *Eimeria* species). On the same day, groups A, B and C were given 1, 2 and 3% of *C. sinensis* essential oil, respectively, in feed, group D with Toltrazuril® while groups E and F were left unmedicated. The results revealed *C. sinensis* essential oil to have positive effect on FCR in broilers. Moreover, there was a significant improvement in oocysts per gram of faeces, lesion score, oocyst score and faecal score in oil treated broilers at the maximum inclusion level of 3% comparable to the standard control. However, apart from significant effect on WBCs count and serum levels of ALT and LDH, *C. sinensis* essential oil had very limited non-significant effect on hematological and serum biochemical parameters, and organs' weight. Thus, the present study provided *C. sinensis* essential oil to be an alternate anticoccidial agent which can be used in coccidiosis control programs after further validation.

To Cite This Article: Imran A and Alsayeqh A, 2022. Anticoccidial efficacy of *Citrus sinensis* essential oil in broiler chicken. Pak Vet J, 42(4): 461-466. http://dx.doi.org/10.29261/pakvetj/2022.082

INTRODUCTION

In recent years, poultry industry is growing rapidly in most parts of the world and is the milestone in the provision of quality and cheaper protein in the scenario of rapidly growing human population. However, food security is always compromised by the attack of various infectious diseases on poultry such as coccidiosis (Khan *et al*., 2021; Saeed 2021). Coccidiosis is a protozoal disease caused by species of genus *Eimeria* and is highly fatal to poultry resulting in huge economical losses (Abbas *et al.,* 2017a; Martins *et al.,* 2022). It primarily infects the intestinal tract of poultry, causing high morbidity and mortality. The disease transmits through faecal-oral route by ingesting coccidial oocysts**,** leading towards enteritis, bloody diarrhea, poor growth, emaciation and ultimately death (Abbas *et al.,* 2020; Mohsin *et al.,* 2021a & b). Different species of *Eimeria* affect poultry like *Eimeria (E.) tenella*, *E. necatrix*, *E. maxima*, *E. mitis*, *E. brunette* and *E. acervulina*. Various factors predisposing poultry to this fatal disease include wet litter, poor management and high stocking density (Khater *et al.,* 2020). It also compromises the immunity of poultry, thus, increasing chances of other secondary infections (Rizwan *et al.,* 2022).

Different methods are used for the control of coccidiosis in poultry. However, the most common and traditional approach is chemical control through different anticoccidial drugs (Khater *et al.,* 2020). But this method is becoming obsolete due to the emergence of drug resistant *Eimeria* species (Abbas *et al.,* 2017a). Apart from this, the other control methods also have some limitations. For example, the vaccination method is expensive as well as risky owing to chances of vaccination failure (Chapman, 2014; Kalkal *et al.,* 2021). Hence, other effective alternatives are required to overcome the challenge of infectious diseases including coccidiosis in poultry (Mohsin *et al*., 2021 a & b; Nazir *et al*., 2021). Among the alternatives, botanical compounds are finding an increasing use in coccidiosis control programs owing to their promising results (Wajiha and Qureshi, 2021; Abbas *et al.,* 2022). These botanical compounds when co-administered with vaccine, boosted the vaccine response in chicken (Ritzi *et al.,* 2016). These botanicals may be either plant extracts or essential oils.

Different essential oils like oregano, garlic and others have been shown to have anticoccidial properties (Idris *et al.,* 2017; Sidiropoulou *et al.,* 2020). These essential oils contain a variety of bioactive compounds which impart medicinal properties to them. However, the anticoccidial effect of *C. sinensis* essential oil has not yet been investigated. Hence, the current *in-vivo* experiment was designed to evaluate the anticoccidial potential of *C. sinensis* essential oil in broilers.

MATERIALS AND METHODS

Procurement and analysis of essential oil: *C. sinensis* essential oil was purchased from a commercial market in Faisalabad, Punjab, Pakistan and was used in pure form for the *in-vivo* trial. The oil was subjected to GC-FID analysis using GC-17, Shimadzu apparatus at the Central Hi-Tech. Laboratory, University of Agriculture, Faisalabad, Pakistan for determining its chemical composition. The resultant peaks were expressed as percentages of the corresponding components in the oil.

Collection and sporulation of *Eimeria* oocysts: Coccidiosis-suspected broiler guts were obtained from meat shops and broiler farms in Faisalabad. After collection, the guts were brought to the laboratory, cut-opened and the gut contents collected. These contents were then examined under the microscope, and the confirmed *Eimeria*-positive contents were preserved in 2.5% Potassium dichromate solution. Furthermore, using the technique of Ryle *et al.* (1976), the *Eimeria* oocysts were sporulated and isolated.

***In vivo* experiment:** For the *in-vivo* experiment, 72 chicks (one day old) were selected and raised according to the recommended industry standards. During the trial, chicks were fed a mash meal that was free of coccidiostats. On day 7, chicks were randomly divided into six groups named A, B, C, D, E and F, with each group having 12 chicks. On14th day, all the groups except F were orally given essential oil as well as 50000 sporulated oocysts of mixed *Eimeria* species. Groups A, B and C were supplied a feed that was mixed with the *C. sinensis* essential oil at 1, 2 and 3% concentrations, respectively, for three consecutive days. Group D served as positive control that was provided with both the infection and the Toltrazuril® treatment. Group E was serving as negative control that was given infection but not treated with any anticoccidial drug. Group F was the normal control group that was neither given coccidial infection nor treatment.

Evaluation of parameters: The following parameters were evaluated to determine the *in-vivo* anticoccidial activity of *C. sinensis* essential oil.

Mortality and feed conversion ratio: Seven days after the administration of *Eimeria* oocysts to chicks, mortality rate and feed conversion ratio (FCR) were calculated in all the treatment groups. Number of dead birds was recorded and the percent mortality was determined. Likewise, FCR was also determined from the feed consumption and the weight gain by broilers.
The following formulae were used to calculate these parameters:

Mortality = (No. of dead birds/Total No. of birds) × 100
FCR = Feed intake in grams/Weight gain in grams

Oocysts per gram of faeces and lesion scoring: Both the oocysts per gram (OPG) of faeces and the lesion scoring were done seven days post-infection. The McMaster counting chamber was used to determine the OPG following the procedure mentioned by MAFF (1979) while lesions were scored from 0 to 4 following the procedure of Johnson and Reid (1970). OPG was calculated using the following formula:
OPG = Oocysts count-factor (volume of faecal sample/volume of counting chamber)

Oocyst scoring: Oocysts scoring was done for three consecutive days from 5th to 7th day post-infection. Cecal scrapings were obtained, examined under the microscope and scoring was carried out between 0-4 using the procedure described by Long *et al.* (1976).

Faecal scoring: Faecal scoring was done on a scale of 1-5 from the third to the seventh day after inoculation of *Eimeria* oocysts. Normal faeces received a score of 1, whereas a score of 5 indicated severe diarrhea with a significant amount of blood present (Youn *et al.,* 1993).

Organ's weight: On the 35th day of the experiment, each group's remaining chicks were individually weighed and slaughtered. The internal organs of chicks, including the liver, heart, spleen, intestine, gizzard and proventriculus were surgically removed and weighed. The results were expressed in percentages to carcass weights.

Hematological and serum biochemical parameters: On 35th day of the experiment, chicks were slaughtered, and blood was collected to determine different hematological and serum biochemical parameters. In hematological analysis, packed cell volume (PCV), hemoglobin (Hb) concentration and blood cells count were determined using methods of microhematochrit, Sahli's, and Natt and Herrick (1952) respectively whereas serum biochemical parameters like ALT, AST, urea, creatinine and LDH were assessed using Merck kits.

Statistical analysis: The results were interpreted with the help of Duncan's Multiple Range Test and ANOVA, considering the results to be significant at $P<0.05$ having confidence level of 95%.

RESULTS

Essential oil analysis: The GC-FID analysis graph of the *C. sinensis* essential oil is shown in Fig. 1. The detected components along with their retention times and concentrations in percentages are described in Table 1.

Mortality and feed conversion ratio: The effects of *C. sinensis* essential oil on mortality and FCR were found to be dose-dependent. Mortality occurred only in groups A, B and E. Similarly, FCR improved dose-dependently in the groups A, B and C with the results in group C almost identical to that of group D (Table 2). Due to the group feeding of broilers, statistical analysis of the FCR was not practicable.

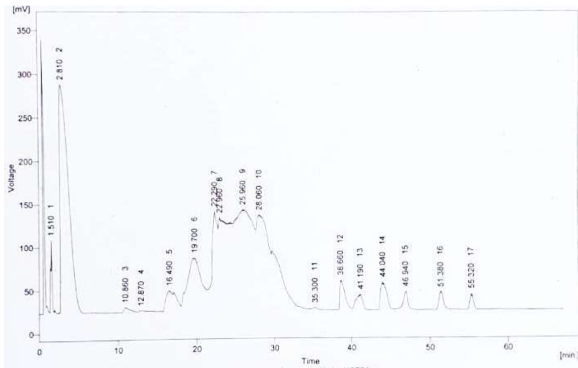

Fig. 1 GC-FID Analysis of *C. sinensis* Essential Oil.

Table 1: *Citrus sinensis* Essential Oil Composition

Component	Retention Time (min.)	Concentration (%)
Acetaldehyde	2.810	20.6
Anisaldehyde	46.940	1.0
Benzaldehyde	22.290	6.4
Citral	28.060	20.3
Ethyl acetate	1.510	1.0
Gamma-terpinene	19.700	8.2
Linalool	25.960	25.7
Nerol	38.660	1.7
Octanal	16.490	2.5
Valerolactone	41.190	1.2
5-methyl furfural	44.040	1.9

Table 2: Effect of different treatments on mortality and feed conversion ratio in broilers

Group	Mortality (%)	FCR
A	7.33	1.96
B	7.33	1.87
C	0.00	1.62
D	0.00	1.58
E	32.55	2.38
F	0.00	1.80

A: *C. sinensis* 1% treated; B: *C. sinensis* 2% treated; C: *C. sinensis* 3% treated; D: Toltrazuril® treated; E: infected untreated; F: uninfected untreated.

Oocysts per gram of faeces and lesion scoring: The effect of *C. sinensis* essential oil on the OPG and lesion score in almost all the treatment groups was observed to be significant ($P<0.05$), as shown in Fig. 2 and Fig. 3. However, the results between the groups C and D were non-significant ($P>0.05$) showing similar results to the standard medicine at 3% addition.

Oocysts scoring: The results of oocysts scoring revealed a dose-dependent response of *C. sinensis* essential oil. The score improved as the essential oil's concentration increased. The results at 3% concentration of *C. sinensis* essential oil in group C were non-significantly different from that of group D ($P>0.05$), as depicted in Table 3.

Faecal scoring: The faecal scoring in the treatment groups on 5th, 6th and 7th day of infection showed dose dependent response with non-significant difference ($P>0.05$) between the groups C and D (Table 4). In groups C and D, the results were comparable, showing non-significant differences with each other ($P>0.05$).

Hematological and serum biochemical parameters: The effect of different treatments on hematological parameters such as RBCs, WBCs, PCV and Hb is shown

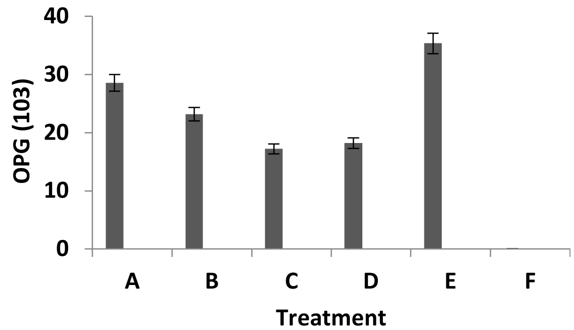

Fig. 2 Effect of different treatments on OPG in broilers. A: *C. sinensis* 1% treated; B: *C. sinensis* 2% treated; C: *C. sinensis* 3% treated; D: Toltrazuril® treated; E: infected untreated; F: uninfected untreated.

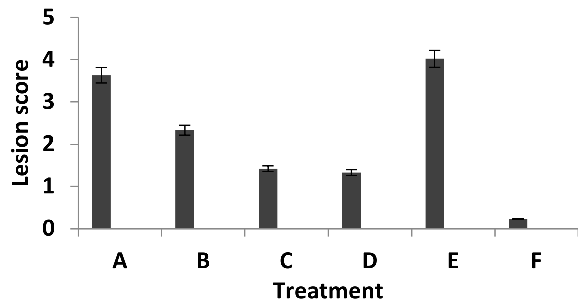

Fig. 3 Effect of different treatments on lesion score in broilers. A: *C. sinensis* 1% treated; B: *C. sinensis* 2% treated; C: *C. sinensis* 3% treated; D: Toltrazuril® treated; E: infected untreated; F: uninfected untreated.

Table 3: Effect of different treatments on oocyst score in broilers

Group	5th day	6th day	7th day
A	3.48±0.72^{a}	3.22±0.52^{a}	3.12±0.81^{a}
B	2.62±0.25^{b}	2.55±0.62^{ab}	2.44±0.68^{b}
C	1.78±0.36^{bc}	1.64±0.26^{d}	1.56±0.98^{cb}
D	1.80±0.78^{c}	1.54±0.11^{c}	1.49±0.71^{c}
E	3.88±0.52^{a}	3.87±0.36^{b}	3.76±0.72^{ab}
F	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}

Means in the same column bearing different superscripts differ significantly. A: *C. sinensis* 1% treated; B: *C. sinensis* 2% treated; C: *C. sinensis* 3% treated; D: Toltrazuril® treated; E: infected untreated; F: uninfected untreated.

Table 4: Effect of different treatments on faecal score in broilers

Group	5th day	6th day	7th day
A	3.87±0.68^{ab}	3.46±0.45^{ab}	3.14±0.48^{ab}
B	2.88±0.48^{b}	2.45±0.78^{b}	2.34±0.18^{b}
C	1.53±0.66^{bc}	1.43±0.58^{bc}	1.36±0.17^{bc}
D	1.61±0.56^{c}	1.45±0.57^{c}	1.46±0.16^{c}
E	4.33±0.54^{a}	4.22±0.51^{a}	4.63±0.58^{a}
F	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}

Means in the same column bearing different superscripts differ significantly. A: *C. sinensis* 1% treated; B: *C. sinensis* 2% treated; C: *C. sinensis* 3% treated; D: Toltrazuril® treated; E: infected untreated; F: uninfected untreated

in Table 5. There was a non-significant difference in WBCs count between groups C and D, while all other results varied significantly among different groups. Similarly, a non-significant difference was observed between groups C and D only for ALT and LDH ($P>0.05$) as in Fig. 4.

Organ's weight: The organs' weight between different treatment groups varied non-significantly ($P>0.05$). This showed that *C. sinensis* essential oil had low or very limited effects on organs' weight (Fig. 5).

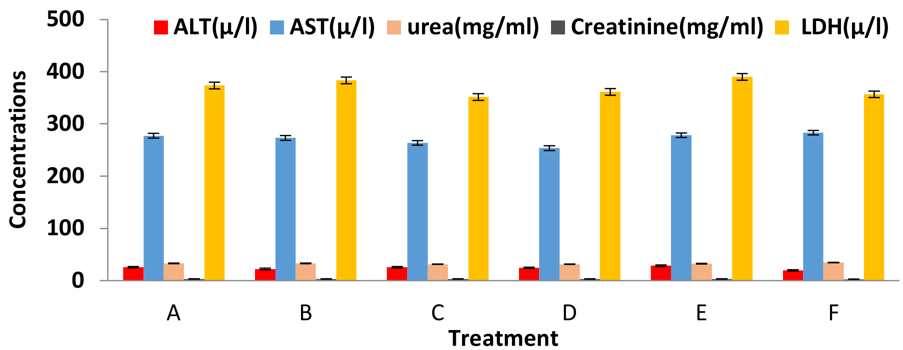

Fig. 4 Effect of different treatments on different serum biochemical parameters in broilers. A: C. sinensis 1% treated; B: C. sinensis 2% treated; C: C. sinensis 3% treated; D: Toltrazuril® treated; E: infected untreated; F: uninfected untreated

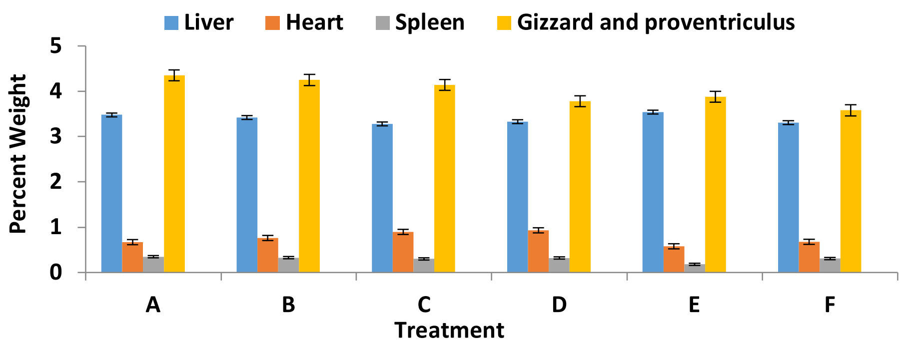

Fig. 5 Effect of different treatments on different organs' weight in broilers. A: C. sinensis 1% treated; B: C. sinensis 2% treated; C: C. sinensis 3% treated; D: Toltrazuril® treated; E: infected untreated; F: uninfected untreated.

Table 5: Effect of different treatments on hematological parameters in broilers

Group	PCV (%)	Hb (g/dl)	RBCs (10^6/µl)	WBCs (10^3/µl)
A	26.39 ± 2.26^{bc}	10.46 ± 3.44^{ab}	2.85 ± 1.23^{bc}	25.68 ± 1.65^{b}
B	27.55 ± 1.57^{a}	10.56 ± 3.56^{a}	2.88 ± 0.28^{c}	25.12 ± 0.86^{c}
C	30.20 ± 9.47^{b}	11.63 ± 5.65^{b}	3.77 ± 0.68^{a}	24.07 ± 9.52^{ac}
D	29.70 ± 15.63^{c}	11.53 ± 3.75^{c}	3.68 ± 0.54^{c}	23.05 ± 6.25^{a}
E	20.48 ± 0.97^{a}	9.35 ± 0.54^{a}	1.75 ± 0.25^{cb}	32.53 ± 11.58^{bc}
F	31.60 ± 4.39^{c}	11.70 ± 5.98^{c}	3.59 ± 1.75^{a}	26.10 ± 4.58^{ca}

Means in the same column bearing different superscripts differ significantly. A: *C. sinensis* 1% treated; B: *C. sinensis* 2% treated; C: *C. sinensis* 3% treated; D: Toltrazuril® treated; E: infected untreated; F: uninfected untreated.

DISCUSSION

Coccidiosis is an important parasitic disease inflicting huge losses on the poultry industry. Most commonly, chemical drugs have been used for its control. However, due to the emerging issues of public safety and resistance development, there is an increasing shift towards botanicals as alternatives (Abbas *et al.,* 2012; 2017b; 2019). These botanicals provide better control than commercially used anticoccidial drugs (Grandi *et al.,* 2016).

Among these botanicals, essential oils are very important anticoccidial agents as proved by various *in-vitro* and *in-vivo* studies (Remmal *et al.,* 2011; Jitviriyanon *et al.,* 2016; Sidiropoulou *et al.,* 2020; Langerudi *et al.,* 2022). These are rich in bioactive compounds like polyphenols, polysterols, flavonoids and tocopherols, which impart antioxidant and other therapeutic properties to these oils (Abbas *et al.,* 2013; Jorge *et al.,* 2016). These compounds may act either directly by interrupting the metabolism of *Eimeria* parasites or indirectly by improving the bird's immunity. For example, linalool component of essential oils directly targets the protease activity of parasites (de Almeida *et al.,* 2007). Similarly, as the *Eimeria* leads to generation of reactive oxygen species, these antioxidant compounds help to counter this oxidative stress (Idris *et al.,* 2017).

Like other essential oils, *C. sinensis* essential oil also bears many antioxidant compounds (Jorge *et al.,* 2016). The major compounds as revealed by GC-FID analysis include citral, linalool and acetaldehyde which may be responsible for the anticoccidial effect observed in this current experiment. For example, acetaldehyde has been proven to help fight coccidiosis by inducing early egress of *Eimeria* sporozoites (Yan *et al.,* 2016). Additionally, *C. sinensis* essential oil has also exhibited anticoccidial effect in the *in-vitro* experiment by inhibiting sporulation of *Eimeria* oocysts (Salman and Imran, 2022).

Moreover, the administration of *C. sinensis* essential oil caused an improvement in the FCR of infected broilers. These results are very similar to another study where *C. sinensis* peel supplementation in feed also improved the performance of broilers by increasing weight gain (Ebrahimi *et al.,* 2013). Similarly, improved broiler performance and caecal microbiota were also observed when administered encapsulated citral which is the major component of *C. sinensis* essential oil (Yang *et al.,* 2020). This improvement may be attributed to the positive impact of *C. sinensis* on the gut microflora in broilers which is responsible for feed digestion and maintenance of gut health (Zohreh *et al.,* 2012; Yang *et al.,* 2020).

Furthermore, *C. sinensis* essential oil supplementation lowered oocysts shedding in faeces, lesion score, oocyst score and faecal score. These results agree with previous findings where many essential oils have been proved to exert similar effects due to their anti-inflammatory, antioxidant and anti-protozoal properties (Khater *et al.,* 2020; Chang *et al.,* 2021; Langerudi *et al.,* 2022). These essential oils help in countering the inflammation through enhanced secretion of anti-inflammatory cytokines or inhibited arachidonate metabolism. Moreover, they modulate the intracellular parasite killing response through improved production of nitric oxide inside macrophages (Monzote *et al.,* 2012).

However, *C. sinensis* essential oil did not significantly affect the organ's weight. This finding agrees with previous studies where other essential oils also failed to do so (Kucukyilmaz *et al.,* 2012; Aguilar *et al.,* 2013). However, the significant effect on WBCs count in broilers fed *C. sinensis* essential oil is authenticated by results of a previous study where the administration of orange extract also affected WBCs count significantly (Azizi *et al.,* 2018). This significant effect on WBCs count suggests the immunostimulatory effect of *C. sinensis* essential oil. Similarly, like *C. sinensis* essential oil, many other oils have also been proven to affect the serum levels of various enzymes (Faix *et al.,* 2009; Zhu *et al.,* 2014; Ghanima *et al.,* 2021).

Conclusions: From the findings of the current experiment, it is concluded that *C. sinensis* essential oil bears significant anticoccidial potential at 3% inclusion level. It not only counters the coccidiosis-induced damage but also helps to improve weight gain by helping beneficial gut microflora. However, further trials are still recommended to validate the current results before commercially using this essential oil in coccidiosis control programs.

Authors contribution: Aqsa Imran and Abdullah Alsayeqh designed the experiment. AI executed the research and collected the data. AI and AA analyzed the data and wrote the manuscript.

Acknowledgements: The authors would like to thank the Deanship of Scientific Research, Qassim University, Saudi Arabia for funding the publication of this project.

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