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In Vitro Evaluation of Oocysticidal and Sporulation Inhibition Effects of Essential oil of Orange (*Citrus sinensis*) Against *Eimeria tenella*

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ABSTRACT

A study was carried out to assess the oocysticidal and sporulation inhibition effects of essential oil (Eos) of orange (*Citrus sinensis*) against oocysts of *Eimeria tenella by in vitro* assay. Essential oil was extracted from orange peel by hydro-distillation using Clevenger apparatus at 80º C for 1-2 hours. In the *in vitro* trials, 20, 30, and 40 per cent of working concentration, prepared from essential oil and ethanol mixture (1: 5 ratio stock solutions), were tested against freshly collected oocysts of *Eimeria tenella*. The results showed that 57.2 ± 0.14, 64.0 ± 0.18 and 42.2 ± 0.25 per cent of the sporulated oocysts and 6.8 ± 0.27, 19.3 ± 0.35 and 36.0 ± 0.33 per cent of unsporulated oocysts were found to have been damaged in the 20, 30 and 40 percent concentration of essential oil respectively. In the positive control group (1% Sodium hypochlorite), 78.0 ± 0.03 and 17.3 ± 0.21 per cent of sporulated and unsporulated oocysts, respectively were found damaged. While 81.31 ± 0.06 and 80.3 ± 0.03 per cent oocysts got sporulated and did not show any damage in the negative control groups (50 % Ethanol and water). The highest sporulation inhibition of 43.7 ± 0.15 per cent was recorded in the 40 per cent group.

HIGHLIGHTS

- Coccidiosis is one of the most common diseases worldwide and caused by *Eimeria* species.
- *Citrus* based essential oil having oocysticidal and sporulation inhibition effect.
- Essential oil of orange peel (30 %) can be used as organic disinfectant in broiler farms.

Keywords: *Citrus sinensis*, Essential oil, *Eimeria tenella,* GC-MS, *In vitro,* oocysticidal activity

Poultry industry is one of the fastest growing sectors of agriculture in India today. However, outbreak of various diseases is the major impediment to the profitable farming, particularly in commercial broiler production. Among the economically important diseases of broiler chicken, coccidiosis still continues to be one of the most common diseases worldwide (Mc Douglad, 1997). The global economic loss due to this disease is estimated to be 800 million dollar per annum (Yousuf and Tak, 2013) and in India, economic loss due to coccidiosis is 14 billion (Bera *et al.,* 2010). Although the current strategy of using anticoccidials and vaccines to control coccidiosis is cost effective and successful, the presence of drug residue resistance and public demand for residue free meat has encouraged development of alternative control strategies (Muthamilselvan *et al.,* 2016). The importance of essential oil in poultry production is increasing due to their therapeutic properties against various diseases. Coccidiostatic effect of oregano essential oil against *E tenella* and anticoccidial activity of *Citrus* based essential

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oil against sheep coccidiosis by decreasing coccidia invasion were reported (Giannenas *et al.,* 2004 and Dudko *et al*., 2018). Coccidiosis is transmitted by ingestion of sporulated oocysts, which are able to survive for longer period in litter and environment. Hence, farmers usually use disinfectants such as ammonia, methyl bromide, carbon disulphide and some phenolic compounds to destroy the oocysts before arrival of each new batch of chicks, but these compounds may not ensure complete destruction, leading to occurrence of the disease. In addition, persistence of toxicity of chemical disinfectants in the environment may harm the birds and human health and hence, it is the need of the hour to develop environment friendly oocysticidal product of plant origin. Although Remmal *et al.* (2013) reported that essential oils were efficient to destroy *Eimeria* oocysts under *in vitro* condition in 2011, since then it has not drawn much attention and only few works have been carried out in different parts of the world. Since Namakkal and surrounding districts in Tamil Nadu are known for commercial layer and broiler production, a study was undertaken to determine the oocysticidal and sporulation inhibition effects of essential oil of orange.

MATERIALS AND METHODS

Extraction of essential oil

Essential oil was extracted from orange peel by hydrodistillation as per method described by (Radunz *et al.*, 2002). The peels were cut into small pieces and approximately 100 g of cut peel was pulverized in domestic mixer and placed into 1000 ml boiling flask to which 500 ml of water was added. The flask with pulverized peel was placed on heating mantle and then carefully fixed with Clevenger apparatus. The Clevenger apparatus was then connected with tap water to ensure continuous circulation of water during extraction of oil. The contents were boiled at 80º C for 2 hrs and the essential oil collected above the water in the graduated collecting tube was drained into glass vials and stored at 4º C until further use.

Chemical analysis of essential oil

The essential oil that was extracted from orange peel was submitted to National Institute of Food Technology Entrepreneurship and Management, Thanjavur, Tamil Nadu, India for chemical analysis. The sample was extracted with ethyl acetate and analyzed through Gas Chromatography-Mass Spectrometry using equipment TSQ 9000 Triple Quadruple Mass spectrometer for identification of different compounds. The carrier gas flow managed at a rate of 1 ml /min. The temperature programme was 110º C for 3.50 min, 200º C at the rate of 10º C / min – no hold and up to 280º C at the rate 5º C / min - 12 min hold and injector temperature was 280º C and total GC running time was 40 - 50 min. The mass spectra were obtained at an ionization voltage of 70 eV. The identification of compounds in the chromatographic profile was achieved by comparison of their mass spectra with a library data base (NIST version -2011).

Propagation of *Eimeria tenella* oocysts

A total of 15 numbers of 21 day old broiler chickens were procured from commercial farm. All the birds were inoculated with sporulated oocysts of *Eimeria tenella* at the rate of 6.6×10^3/ bird on 28th day of age and monitored regularly. On the 6th day of infection bloody droppings were observed. On 9th day, one bird was sacrificed and caecal contents were collected to harvest oocysts for *in vitro* trial .The number of oocysts in 1 ml of caecal suspension was determined using Mc Master counting chamber and then diluted with distilled water so as to contain approximately 200 oocysts in 100 µl of suspension.

Preparation of working concentration of Essential oil

Stock solution was first prepared by mixing 1.5 ml of orange essential oil with 7.5 ml of Ethanol at the ratio of 1:5. After that 2, 3 and 4 ml of stock solution was transferred to three different vials containing 8, 7 and 6 ml of distilled water to obtain 20, 30 and 40 per cent of working concentration respectively.

In vitro trial

Efficacy of essential oil against *E. tenella* oocysts

In this trial 100 µl of oocysts suspension containing approximately 200 number of oocysts was pipetted into each well in the first row of 24 well tissue culture plate, to which 500 µl of 20 per cent Eos followed by 400 µl

of distilled water were added to maintain 0.5 cm depth, providing ideal condition for sporulation. Similarly the wells in the second and third rows were allotted for 30 and 40 per cent Eos. While in the fourth row 2 wells each were used for positive control (1% Sodium hypochlorite) and negative control (water and 50% Ethanol). The plate was then kept at room temperature with periodical shaking for 24 hours. After 24 hours, a total of hundred numbers of oocysts, which included sporulated damaged, sporulated normal, unsporulated damaged and unsporulated normal were counted under the inverted tissue culture microscope (Leica, type-090-135.001) in order to determine the oocysticidal and sporulation inhibition effect of orange Eos. The oocysticidal and sporulation inhibition effects of essential oil was determined based on the difference in the percentage of oocysts damaged (sporulated and unsporulated) and percentage of oocysts remained normal (sporulated and unsporulated) between treatment and control groups (positive and negative). However, Abbott's formula was used to arrive actual percentage of sporulation inhibition in treatment group by correcting percentage oocysts that remained unsporulated in the control groups.

$$\text{Inhibition (\%)} = \frac{X - Y}{X} \times 100$$

(X = Percent sporulated in the control, Y = Percent sporulated in treatment group).

STATISTICAL ANALYSIS

The data collected were analyzed by Chi square method (McHugh, 2013).

RESULTS AND DISCUSSION

In the present study, 100 g of orange peel yielded maximum of 2.5 - 5.0 ml of essential oil. This observation is not in agreement with the findings of Cholke *et al.* (2017) who reported that 10 ml essential oil was extracted from 100 g of the orange peels of C*itrus sinensis.* The difference in the oil yielding may be due to temperature and duration of extraction, besides stage of fruit, soil type and climatic condition of the locality from where fruits originated.

The chemical compounds identified in the essential oil of *Citrus sinensis* are presented in (Table 1). Gas chromatography analysis revealed the presence of 27 different compounds of which 6 compounds such as D-Limonene (5.61%), ç-Terpinene (6.17%), Geraniol (7.77%), Phenol,5-methyl-2-(1- methylethyl)- (6.20%), Bis (2-ethylhexyl) phthalate (10.90%) and 1,2-Benzenedicarboxylicacid, dinonyl ester (16.18%) were found to be in higher proportion. The findings of the present study are not in corroboration with the findings of Okunowo *et al*. (2013) and Cholke *et al.* (2017) who recorded D-Limonene (65%) as the major component of citrus essential oil. However, a marked variation in the percentage proportion of D-Limonene might be due to method employed for oil extraction and phytochemical analysis.

Table 1: Compounds identified in the essential oil of orange

No	Retention Time (Min)	Name of the compound	Peak Area %
1	3.13	D-Limonene	5.61
2	3.40	ç-Terpinene	6.17
3	3.75	1,6-Octadien-3-ol, 3,7-dimethyl-	3.90
4	4.40	*cis*-Limonene oxide	1.63
5	4.54	6-Octenal, 3,7-dimethyl-	2.23
6	5.28	à-Terpineol [à,alpha.,4-trimethyl-3-cyclohexene-1-methanol]	2.68
7	5.42	Decanal	4.82
8	5.77	Citronellol	1.78
9	6.07	Neral	2.80
10	6.30	Geraniol	7.77
11	6.57	2,6-Octadienal, 3,7-dimethyl-, (E)-	3.68
12	6.89	Phenol, 5-methyl-2-(1- methylethyl)-	6.20
13	7.06	Carvacrol	4.25
14	8.32	2,6-Octadien-1-ol,3,7-dimethyl-, acetate, (E)-	1.37
15	8.38	Copaene	1.44
16	9.07	Caryophyllene	2.21
17	9.56	Humulene	1.09
18	9.83	ç-Muurolene	0.07
19	10.15	à-Farnesene	1.92
20	10.39	Citronellyl butyrate	0.58
21	10.46	ë-Cadinene	2.16
22	10.84	Neryl butyrate	6.73
23	12.15	à-Cadinol	0.64
24	15.63	n-Hexadecanoic acid	0.92
25	17.91	9,12-Octadecadienoic acid (Z,Z)-	0.27
26	23.89	Bis(2-ethylhexyl) phthalate	10.90
27	26.97- 28.64	1,2-Benzenedicarboxylicacid, dinonyl ester	16.18

Table 2: Oocysticidal effect of essential oil of *Citrus sinensis* against *Eimeria tenella* oocysts

Parameter	Treatment			Control		
	20%	30%	40%	Sodium hypochlorite (1%)	Ethanol (50%)	Water
Percentage of damaged sporulated oocysts (Mean ± SE)	57.2 ± 0.14	64 ± 0.18	42.2 ± 0.25	78 ± 0.03	0	0
Percentage of undamaged sporulated oocysts (Mean ± SE)	21.2 ± 0.33	10.5 ± 0.17	3.0 ± 1.6	2.3 ± 1.0	81.31 ± 0.06	80.3 ± 0.03
Percentage of damaged unsporulated oocysts (Mean ± SE)	6.8 ± 0.27	19.3 ± 0.35	36.0 ±0.33	17.3 ± 0.21	0	0
Percentage of undamaged unsporulated oocysts (Mean ± SE)	14.8 ± 0.11	19.5 ± 0.31	17.2 ± 0.28	2.3 ± 0.65	18.6 ± 0.26	19.6 ± 0.15

Table 3: Effects of essential oil of *Citrus sinensis* on sporulation of *Eimeria tenella* oocysts

Concentraion of oil	Treatment		Actual % of sporulation inhibition Abbott's formula = $\frac{X-Y}{X}\times 100$	Control					
				Sodium hypochlorite (1%)		Ethanol (50%)		Water	
	Percentage of Sporulated oocysts	Percentage of Un Sporulated oocysts		Percentage of Sporulated oocysts	Percentage of Un Sporulated oocysts	Percentage of Sporulated oocysts	Percentage of Un Sporulated oocysts	Percentage of Sporulated oocysts	Percentage of Un Sporulated oocysts
20%	78.4 ± 0.01	21.6 ± 0.06	2.3 ± 0.01	80.3 ± 0.06	19.6 ± 0.25	81.4 ± 0.06	18.6 ± 0.26	80.3 ± 0.03	19.6 ± 0.15
30%	74.5 ± 0.15	38.8 ± 0.26	7.2 ± 0.15	—	—	—	—	—	—
40%	45.2 ± 0.30	53.2 ± 0.17	43.7 ± 0.30	—	—	—	—	—	—

In the present study, oocysts incubated with different concentration of essential oil i.e., 20, 30 and 40 per cent showed damage in the form of cracked and corrugated / dented oocyst wall, bleb formation on the wall, contents expelled empty oocysts and disfigured and crumbled oocysts. These findings are in accordance with Remmal *et al.*, 2013 who observed deformed oocysts with cracked wall and debris while studying the *in vitro* oocysticidal effects of essential oil of orange. Similarly, Cacho *et al.* 2010 reported that oocyst wall formation was significantly altered when chicken was treated with artemisinin for coccidiosis. They also observed dose dependent death of developing oocysts and reduced sporulation rate.

The results of the present study are also in agreement with Jitviriyanon *et al*. (2016) who reported that essential oils of *Boesenbergia pandurata* and *Ocimum basilicum* had strong sporulation inhibition activity and also produced a higher ratio of degenerated oocysts.

The results of oocysticidal property of orange essential oil are presented in the Table 2. The results revealed that 57.2 ± 0.14, 64.0 ± 0.18 and 42.2 ± 0.25 per cent of sporulated oocysts and 6.8 ± 0.27, 19.3 ± 0.35 and 36.0 ± 0.33 per cent unsporulated oocysts were found damaged in 20, 30 and 40 per cent concentration of essential respectively. It is worthy to mention that a higher percentage of damage among the sporulated oocysts was recorded in 20 and 30 per cent concentrations, whereas 40 per cent caused damage before oocysts could sporulate. In addition, 30 per cent essential oil caused damages to the sporulated and unsporulated oocysts together up to 83 per cent, while 40 per cent was able to damage 78 per cent, of which 43.7 ± 0.30 per cent of oocysts remained unsporulated. This observation points to dose dependent oocysticidal effects of essential as opined by Cacho *et al.* (2010). A highly significant ($P < 0.01$) difference in the percentage of oocysticidal effects observed between essential oil treated groups and negative control groups (50 % Ethanol

and water) and also between different concentrations of essential oil, while it was almost on parallel with positive control (1% Sodium hypochlorite).

The oocysticidal properties of essential oil could be ascribed to the presence of compounds such as D-Limonene (5.61%), ç-Terpinene (6.17%), Carvacrol (4.25 %), Caryophyllene (2.21 %) and Phenol, 5-methyl-2-(1-methylethyl)- (6.2 %) in the essential oil of orange. This observation of the present study confirms the findings of Remmal *et al*. (2013) who opined that orange Eos is mixture of many components and major oocysticidal effect was due to D-Limonene, ç-Terpinene, Phenol,5-methyl-2-(1-methylethyl), carvacrol and Caryophyllene. Further, a higher percentage of damage among the sporulated could be due to the weakening of oocyst wall following sporulation due to utilization of part of inner wall for formation of sporocyst wall. Jitviriyanon *et al.* (2016) also reported that essential oil of *Boesenbergia pandurata* and *Ocimum basilicum* produced 45.55 and 53.13 per cent oocysticidal effect respectively. The percentage of oocysticidal effect observed by the authors is lower than the present study. This variation in the oocysticidal effect might be due to the plant species from which oils were extracted. In the positive control group (1% Sodium hypochlorite) 78.0 ± 0.03 per cent of the oocysts were completely damaged and degenerated, leaving behind sporocysts only. This finding does not corroborate with the findings of Jatau *et al. (*2017) who reported that commercial disinfectant like sodium hypochlorite, saponated cresol, cresol and formaldehyde solution at the concentration of twice the manufacturer's recommended disinfectant concentration (MRDC) showed low oocysticidal efficacy *i.e*., 20.4 ± 0.80, 32.3 ± 1.70, 20.8 ± 1.20 and 14.3 ± 0.70 percent, respectively. Increasing the concentration to four times MRDC significantly improved oocysticidal effect to 39.0 ± 3.0, 50.2 ± 3.80, 59.9 ± 0.70 and 30.0 ± 1.70 per cent respectively.

In the negative control (Ethanol 50 % and water), none of the oocysts showed any damage. It confirms the findings of Jitviriyanon *et al.* (2016) who observed that the oocysts treated with ethanol did not show damage. As for sporulation inhibition potency of essential oil, the highest concentration i.e., 40 per cent inhibited sporulation in 43.7 ± 0.30 per cent of oocysts as against no sporulation inhibition in control group treated with 1 % sodium hypochlorite and 50 % Ethanol. This observation is not in agreement with the results of You (2014) who reported that cresol inhibited sporulation up to 80.5 per cent. This might be due to higher concentration of cresol which could have caused higher inhibition. Similarly, Gadelhaq *et al.* (2017) stated that sodium hypochlorite showed a significant degree of sporulation inhibition to 49.67 percent, while Ethanol (70%) showed 100 per cent sporulation inhibition. Varying level of sporulation inhibition observed by the authors using sodium hypochlorite and ethanol could be attributed to higher concentration used in their study.

CONCLUSION

The results of the present study showed that 30 per cent essential oil caused damages to the sporulated and unsporulated oocysts together up to 83 per cent, while 40 per cent was able to damaged 78 per cent, of which 43.7 ± 0.30 per cent remained unsporulated. It can be concluded that 30 per cent essential of orange (*Citrus sinensis*) can be used as disinfectant in broiler industry to destroy coccidian oocysts, thereby harmful effects caused by chemical disinfectants to the birds, human and environment can be minimized to the large extent.

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REFERENCES

Allen, P.C., Lydon, J. and Danforth, H.D. 1997. Effects of components of *Artemisia annua* on coccidia infections in chickens. *Poult. Sci*., **76**: 1156–1163.

Bera, A.K., Bhattacharyaa, D., Pana. D., Dharab, A., Kumar, S. and Das, S.K. 2010. Evaluation of economic losses due to coccidiosis in poultry industry in India. *Agric. Econ. Res. Rev*., **23**: 91-96.

Cacho, E. Gallego, M., Francesch, M., Quílez, J. and Sánchez-Acedo, C. 2010. Effect of artemisinin on oocyst wall formation and sporulation during *Eimeria tenella* infection. *Parasitol. Int*., **59**: 506–511.

Cholke, P.B., Bhor, A.K., Shete, A.M. and Sonawane R.K. 2017. Extraction and GC-MS analysis of orange (*Citrus sinensis)* peel oil. *Res. J. Life Sci.,* **2**(5): 41.

Dudko, P., Junkuszew, A., Bojar, W., Milerski, M., Szczepaniak, K., Scouarnec, J.L., Schmidova, J., Tomczuk, K. and Grzybek, M. 2018. Effect of dietary supplementation with preparation comprising the blend of essential oil from *Origanum vulgare* (lamiaceae) and *Citrus* spp. (citraceae) on coccidia invasion and lamb growth. *Ital. J. Anim. Sci*., **17**(1): 57-65.

Gadelhaq, S.M., Waleed M.A. and Shawky, M.A. 2018. In vitro activity of natural and chemical products on sporulation of *Eimeria* species oocysts of chickens. *Vet. Parasitol.,* **251**: 12-16.

Giannenas, I., Florou-Paneri, P.C., Papazahariadou, M., Botsoglou, N., Christaki, E. and Spais, A.B. 2004. Effect of diet supplementation with ground oregano on performance of broiler chickens challenged with *Eimeria tenella*., *Archiv. Fur. Geflugelkunde.* **68**(6): 247-252

Jatau, I.D., Aremu, S.F., Magaji, Y. and Babashani, M. 2017. Oocysticidal effects of some commercial poultry house disinfectants against sporulated *Eimeria tenella* oocysts. *J. Anim. Prod. Res.*, **29**(2): 281-288.

Jitviriyanon, S., Phanthongc, P., Lomaratc, P., Bunyapraphatsarac, N., Porntrakulpipatd, S. and Paraksae, N. 2016. *In vitro* study of anti-coccidial activity of essential oils from indigenous plants against *Eimeria tenella*. *Vet. Parasitol*., **228**: 96–102.

McDougald, L.R., Fuller, L. and Mattiello, R. 1997. A survey of coccidia on 43 poultry farms in Argentina. *Avian. Dis.,* **41**: 923-929.

McHugh, M.L. 2013. The Chi-square test of independence. *Biochemia. Medica.*, **23**(2): 143-9.

Muthamilselvan, T., Kuo, T.F., Wu, Y.C. and Yang, W.C. 2016. Herbal remedies for coccidiosis control: A review of plants, compounds, and anticoccidial actions. *Evid. Based Complementary Altern. Med.,* **2016**: 19.

Okunowo, W.O., Oyedeji, O., Afolabi, L.O. and Matanmi, E. 2013. Essential oil of grape fruit (*Citrus paradisi*) peels and its antimicrobial activities. *Am. J. Plant Sci.,* **4**: 1-9.

Radunz, L.L., Melo, L.C., Berbert, P.A., Barbosa, L.C., Rocha, A.R.P. and Grandi, A.M. 2002. Effect of the temperature of drying in the amount and quality of *Lippia sidoides* Cham of essential oil. *R. Bras. Armaz.,* **27**: 2-9.

Remmal, A., Achahbar, S., Bouddine, L., Chami, F. and Chami, N. 2013. Oocysticidal effect of essential oil components against chicken *Eimeria* oocysts. *Int. J. Vet. Med.,* **2013**: 8.

You, M.J. 2014. Suppression of *Eimeria tenella* sporulation by disinfectants. *Korean J. Parasitol.,* **52**(4): 435-438.

Yousuf, M. and Tak, H. 2013. Coccidiosis in poultry: A review on prevalence, prevention and control. *Int. J. Rec. Sci. Res.,* **4**: 943-945.