In vitro anticoccidial effects of Olive Leaf (*Olea europaea* L. var. Chemlal) extract against broiler chickens *Eimeria* oocysts

Nedjima Debbou-Iouknane[1], Cristina Nerín[2], Meriem Amrane-Abider[3], Abdelhanine Ayad[1]
[1]Department of Environment Biological Sciences, Faculty of Nature and Life Sciences, University of Bejaia, Algeria.
[2]Aragón Institute for Engineering Research (I3A), University of Zaragoza, Campus, Rio Ebro, María de Luna 3, Spain.
[3]Applied Biochemistry Laboratory, Faculty of Nature and Life Sciences, University of Bejaia, Algeria.

Keywords: *Olea europeaL., var. Chemlal; olive leaves; anticoccidial activity; chickens; in vitro.*

Abstract. *The aim of the present study was to evaluate in vitro the anticoccidial activity of olive leaves (OL) on the destruction of Eimeria oocysts isolated from naturally infected chickens. The identification of phenolic compounds was obtained by ultra-high-performance liquid chromatography-mass spectrometry with electrospray ionization. The treatment of Eimeria oocyst with the OL extract and standard compounds (quercetin and oleuropein) leads to their lysis as shown by the release of substances absorbing at 273 nm. The results indicated that the optimum OL extract and the standard compound showed that the number of oocysts decreased after treatment. The OL extract at a concentration of 1.46 mg/mL recorded a decrease rate of 5.89% of Eimeria oocysts after 7-h treatment. On the other hand, quercetin was most effective (48.57%) followed by oleuropein (27.53%) after 7-h treatment. The ethanol treated Eimeria suspension was significantly ($P \leqslant 0.05$) higher than the tested concentration of the OL extract and the standard compound. The findings of the present study showed that it could be concluded that the OL extract possesses the ability to destroy Eimeria spp. In future, in vivo investigations are required to assess the efficiency of the OL bioactive compounds in broiler chickens.*

Introduction

Coccidiosis is defined as an enteric parasitic pathology of birds between the ages of 3 and 18 weeks caused by the protozoa *Eimeria* spp. belonging to the genus *Eimeria* (Metwaly et al., 2012). It is one of the most common and important diseases that has a negative impact on the growth of poultry industry, resulting in about $1.5 billion losses in poultry industry annually worldwide (Arabkhazaeli et al., 2014). This infection causes reduced feed efficiency, body weight gain, and temporary reduction in egg production.

Anticoccidial drugs in feed and water of chickens have a considerable success. The major problem observed of the commonly available anticoccidial drugs is development of resistance in *Eimeria* species, huge residual effects of anticoccidial drugs in meat and toxic effects of disinfectants. There is an intense need to establish new successful treatments to control coccidiosis in poultry farms and to search for alternative environmentally friendly anti-*Eimeria* agents. Surveys have been published that some botanicals have been reported for their promising results as anticoccidials and improving poultry performance worldwide in broiler chickens (Abbaset al., 2013).

In Algeria, *Olea europea* L. (var. Chemlal) is the most dominant olive variety, representing nearly 45% of the national oil production. Olive tree pruning produces a huge quantity of olive leaves (~25 kg/tree). It has been estimated that approximately 100 g of leaves are present for every kilogram of olives used in oil extraction. It has been shown that dried leaves of the leaf olive (*Olea europea* L.) plant have been used in traditional Algerian medicine for thousands of years.

Many deep studies have shown that the beneficial properties of olive leaves are due to valuable biphenolic compounds. Likewise, the use of whole olive leaves and olive leaf extracts has increased considerably in the pharmaceutical and food industries as food additives. Moreover, they are used for antioxidant (Bulotta et al., 2011), antimicrobial (Pereira et al., 2007), lipid-low (Lee and Lee, 2010), and anticoccidial activity (De Pablo et al., 2010). In addition, other investigations have demonstrated the potential role of antiproliferative and apoptotic effects of olive leaves (Han et al., 2009). However, to the author's knowledge, the anticoccidial activity of olive leaves (*Olea europeaL.*, var. Chemlal) has never been reported. The aim of the present study was to evaluate in vitro the anticoccidial activity of olive leaves (OL) growing in Algeria (area of Bejaia) on the destruction of *Eimeria* oocysts isolated from naturally infected chickens.

Materials and methods

Ethics committee approval

Ethics committee approval was received for this study from the scientific committee of Faculty of Nature and Life Sciences, University of Bejaia, Algeria.

Plant materials

The leaves of olive (*Olea europea* L. var.Chemlal) were collected from Soummam Velley, Bejaia province (Algeria), during March and June 2018. Samples were dried in the shade at room temperature until

Correspondence to Prof. Dr. Abdelhanine Ayad, Department of Environment Biological Sciences, Faculty of Nature and Life Sciences, University of Bejaia, Bejaia 06000, Algeria.
Email: abdelhanine.ayad@univ-bejaia.dz

constant weight was obtained, and then crushed using a traditional grinder. The resulting powder was passed through sieves of a standard 125 µm. Only the fraction with a particle size of ⩽ 125 µm was collected, stored at +4°C in amber bottles and sterilized until used.

Extraction and optimization of total phenolic compounds (TPC) by microwave assisted extraction (MAE)

A domestic microwave oven (2450 MHz, Samsung model NN-S674MF, Kuala Lumpur, Malaysia) was modified in order to extract phenolic compounds from the OL powder. Olive pulp powder (1 g) was stirred manually in aqueous ethanol in preparation for extraction using the MAE system. The MAE parameters were microwave power (700–900 W), extraction time (45–75 s), liquid-solid ratio (20–40 mL/g) and ethanol proportion (20–60%). After that, the extract was centrifuged at 10,000 rpm and filtered through a Buchner funnel lined with Whatman No 3-filter paper, and the supernatant was collected in a volumetric flask. The extract was stored at +4°C until used.

High-performance liquid chromatography-mass spectrometry (HPLC–ESI-MS) analysis

The identification of phenolic compounds of the OL extract was obtained by ultra-high-performance liquid chromatography–mass spectrometry with electrospray ionization (UPLC–ESI-MS) and quadrupole-time of flight detector (QTOF). The equipment was Xevo G2 mass spectrometer consisting of an hexapole, a collision cell and time of flight analyser (QTOF) supplied by Waters (Milford, MA, USA). The electrospray probe was used in the positive (ESI^+) and negative (ESI^-) modes as well as the sensitivity analyzer mode. The mass range considered was from 10 to 1,000 Da. The corona voltage was 2.5 kV for (ESI^+) and 0.5 kV for (ESI^-). The sampling cone voltage was optimized between 20 and 50 V. Finally, 30 V was selected for the screening because more peaks were detected. Other MS parameters were as follows: the source temperature was 150°C, the desolvation gas temperature was 450°C and the desolvation gas flow was 650 Lh^{-1}. The MSE mode was selected for the acquisition, and collision ramp energy from 5 to 40 V was used. MassLynx v.4.1 software (Waters, Milford MA, USA) was used to analyze the samples, and CromaLynx (Waters, Milford MA, USA) was used to deconvolve the spectra.

Evaluation of anticoccidial activity

Eimeria oocysts isolation and purification

An oocysts sample of *Eimeria* spp. was isolated from fresh feces of broilers suffering from coccidiosis in Bejaia area (Algeria). Oocysts were sporulated by placing in 2.5% $K_2Cr_2O_7$solution in the presence of suitable humidity (> 70%) and temperature (28°C). Sporulated oocysts were cleaned and counted using Malassez chamber. The mean number of oocysts per milliliter of the sample was calculated. The identification of *Eimeria* species in chickens was made on the basis of some criteria such as size, shape, presence or absence of micropyle, time of sporulation, intestinal location and appearance and coarse characteristics of intestinal lesions (Carvalho et al., 2011). The percentage of each species in the mixed suspension was approximately 32% *E. acervulina*, 27% *E. tenella*, 15% *E. mitis*, 14% *E. brunetti* and 12% *E. maxima*.

The purification of oocysts was carried out from 1 L phosphate buffered saline (PBS, containing 8 g/L NaCl, 0.2 g/L KCl, 1.13 g/L Na_2HPO_4, $2H_2O$ and 0.2 g/L KH_2PO_4). Neutral substrates containing an inhibitor (streptomycin 1 mg/mL and penicillin V 100 IU) were added to prevent any bacterial evolution, and fluconazole, 17 mg/mL, was used as antifungal. The pH was adjusted to 7.4 and the solution was sterilized by membrane filtration through a 0.2 µm filter. The HBSS (Hanks balanced salt solution) medium was carried out in the laboratory (NaCI, 8.0, KCl 0,4; $CaCl_2$, 0,139; D-glucose, 1,0; Na_2HPO_4, 0,0478; KH_2PO_4, 0, 06 and $MgSO_4$, 0,097 g/L in 1000 mL of distilled water). The solution was sterilized as well as that of the 0.2% agar.

Effects of the leaf extract and standard compounds on the decrease of oocysts number

The activity of the OL extract and standard compounds (quercetin and oleuropein) (Fisher scientific, Fair Lawn, NJ, USA) was determined at a concentration of 18.8 mg/mL in triplicate by incubation at ambient temperature (25ºC) for 24 h (Debbou-Iouknane et al., 2019). The suspension was incubated at different periods of time: 0, 1, 3, 5, 7, and 24 h. One milliliter of the suspension contained 100 µL of washed suspension of *Eimeria* oocysts at $4{,}006x10^6$ oocysts/mL, 700 µL of PBS, and 200 µL of the optimum OL extract.

After incubation, the samples were centrifuged at 320 g for 5 min and the absorbance of the supernatant was measured at 273 nm by spectrophotometer (Shimadzu, model: UV 100 Japan). Then, the percentage of destruction sporulated oocysts was recorded. The LC_{50} value was then inferred from the regression curve. The number of destructions of sporulated oocysts was estimated three times in a cell volume of 1 µL amounts to 4.006×10^4. The ethanol solvent was also used as a negative control.

Effect of the diclazuril sodium on the decrease of the oocysts number

Diclazuril (Algicox10 mg/mL), molecular formula $C_{17}H_8C_{l3}N_4NaO_2$ and anticoccidiosis (Diclosol®, Avico, Arab Industry Veterinary Co, Amman, Jordanie) were

tested in triplicate (0.1, 0.3, 0.5, 0.7 and 1 mg/mL) using the microplate method. The number of oocysts was counted twice in a cell volume of 1 µL amounts to 3.98×10^4oocysts/mL.

Statistical analysis

A statistical analysis was performed by using JMP® Software, version 7.0 (SAS Institute Inc, 2007). The results were expressed in means ± SE. The values were statistically significant when the *P* value was ⩽ 0.05. Inoculums suspension taken at 0, 1, 3, 5, 7 and 24 h on oocysts number was examined by the Student *t* test. The lethal concentration is defined as the concentration that reduces the initial number of sporulated oocysts to 50%.

Results

Fig. 1 represents the chromatogram of the phenolic profile of the OL extract by ultra-high-performance liquid chromatography-mass spectro metry with electrospray ionization (HPLC-ESI-MS). The molecular analysis of molecules revealed the presence of several biophenol classes (Table 1) *viz*. flavonols (luteoline-7-O-glucoside, quercetin, quercetin-3-O-rutinoside, quercetino-O-O-(O-galloyl)-hexoside, phenolic acids (chlorogenic acid), secoiridoids (oleuropein, phenolic alcohol (tyrosol/p-hydroxyphenyl ethanol/p-HPEA), isohamnetinneobavaisoflavone, 2, 3-dihydro-amentoflavone, quercetin-3-O-rutinoside, chlorogenic acid, isorhamnetin 3-O-(6"-O-feruloyl)-glucoside) and diligustilide.

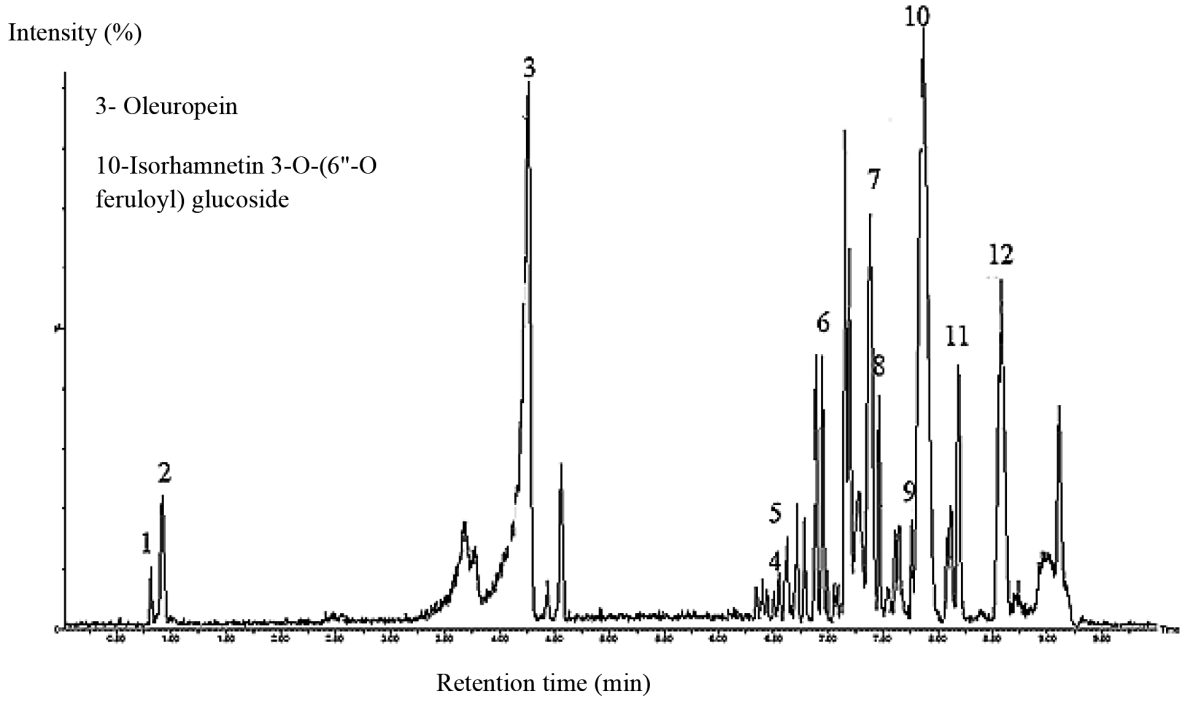

Fig. 1. HPLC–ESI-MS chromatogram ofthe phenolic profile of olive leaves

Table 1. Prediction profilephenolic for the optimal conditions obtained by CCD model

	TR	M-H	µg/g	Name	Formula
1	0.89	447	400,120	Cynarosid/luteolin-7-O-glucoside	$C_{21}H_{20}O_{11}$
2	0.92	137	8261,999	Tyrosol	$C_8H_{10}O_2$
3	4.26	539	75477,543	Oleuropein	$C_{25}H_{32}O_{13}$
4	6.55	301	2023,187	Quercetin	$C_{15}H_{10}O_7$
5	6.63	315	4155,147	Isohamnetin	$C_{16}H_{12}O_7$
6	6.89	321	11056,997	Neobavaisoflavone	$C_{20}H_{18}O_4$
7	7.38	539	36946,034	2, 3-dihydro-amentoflavone	$C_{30}H_{20}O_{10}$
8	7.46	609	7692,308	Quercetin-3-O-rutinoside	$C_{27}H_{30}O_{16}$
9	7.76	706	3140,942	Chlorogenicacid	$C_{16}H_{18}O_9$
10	7.87	688	90257,447	Isorhamnetin 3-O-(6"-O-feruloyl-glucoside)	$C_{32}H_{30}O_{15}$
11	8.19	379	13853,776	Diligustilide	$C_{24}H_{28}O_4$
12	8.58	615	32845,634	Quercetin-o-(o-galloyl)-hexoside	$C_{28}H_{24}O_{16}$

The results indicated that the optimum OL extract and the standard compound, tested at respective concentrations of 44.001 and 18.771 mg/g, showed that the number of oocysts decreased after treatment. The OL extract at a concentration of 1.46 mg/mL recorded a decrease rate of 5.87% of *Eimeria* oocysts after 7-h treatment. On the other hand, quercetin was most effective (56.5%), followed by oleuropein (35.1%) after 7-h treatment. The lethal concentration LC_{50} of the OL extract, quercetin and oleuropein was recorded to be the concentration of 194.92, 14.88 and 173.93 mg/mL, respectively (Fig. 2A, B and C, respectively). Anticoccidial effects of diclazuril were shown as a diminution of the oocysts number

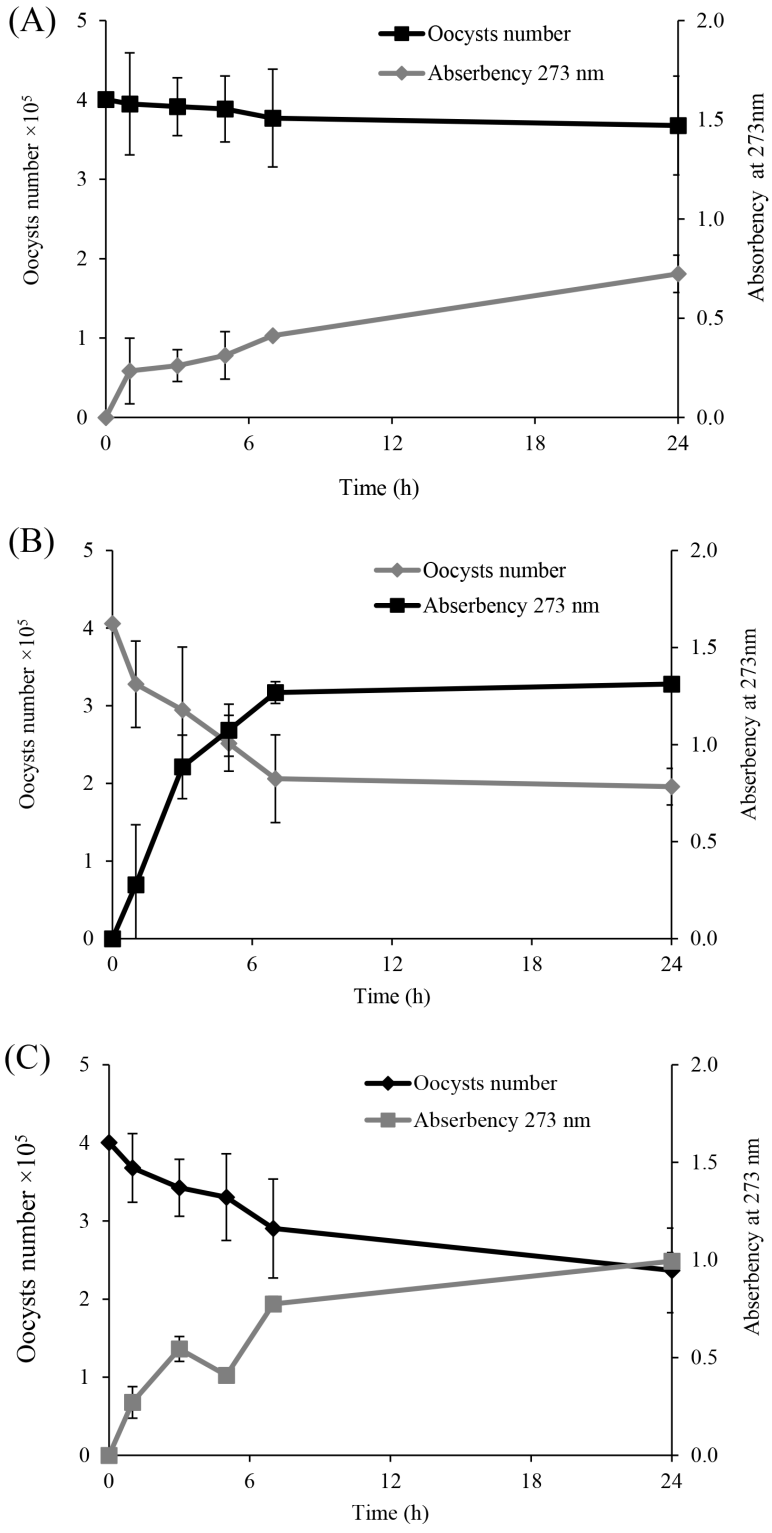

Fig. 2. Kinetics of the decrease of the oocysts number and 273 nm wave length absorbing material release from *Eimeria* oocysts treated by optimum olive leaf extract (*Olea europaea* L., var. Chemlal) (A), quercetin (B), and oleuropein (C)

of 44.3% at 0.5 mg/mL concentration (Fig. 3). The ethanol treated *Eimeria* suspension (negative control) was significantly ($P \leqslant 0.05$) higher than the tested concentration of the OL extract and the standard compound.

According to our results, the OL extract, quercetin and oleuropein were recorded to reduce the oocysts number after7 h for different periods of time (5.89, 48.57 and 27.53%, respectively). This decrease in the number of oocysts causes a considerable release of 273 nm of absorbing material from *Eimeria* oocysts that is depending concentration of the OL extract, quercetin and oleuropein. Fig. 4 (A, B) illustrates a considerable decrease in the number of *Eimeria* oocysts with an increase in the concentration of quercetin and oleuropein ranging from 0.1 to 1 mg/mL.

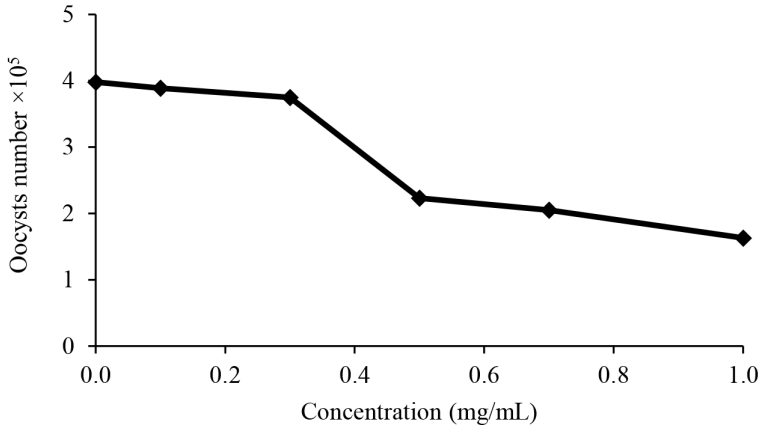

Fig. 3. Effect of diclazuril concentrations on the oocysts number

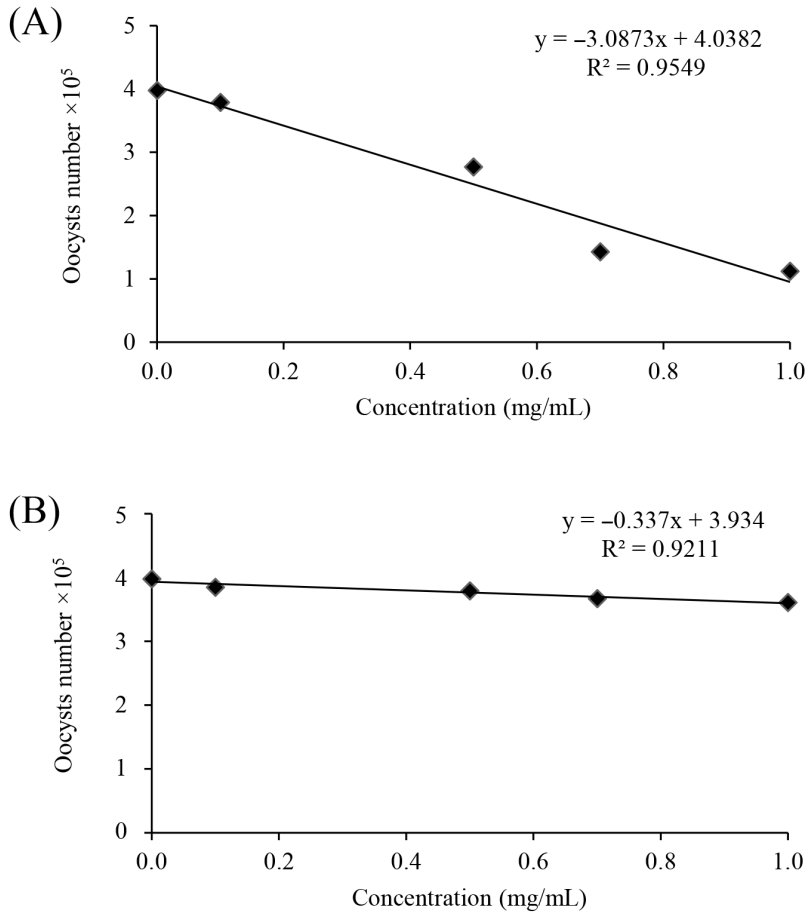

Fig. 4. The correlation between quercetin (A) and oleuropein (B) concentrations and the number of *Eimeria* oocysts

Discussion

Due to the emergence of resistance and drug residues, the use of plant extracts with biological activity has become particularly important to restrict the use of synthetic compounds against coccidial infections (Abbas et al., 2012). Therefore, the use of co-products as an anticoccidial remedy is promising as an alternative for controlling coccidiosis in poultry. Recently, one study has demonstrated the effect of phytochemicals of plant extracts on suppression of *Eimeria* species (Muthamilselvan et al., 2016). The present study work was carried out in order to find a phytobiotic alternative based on an olive co-product to help minimize gastrointestinal disorders caused by *Eimeria* coccidial infection in broiler chickens. To the author's knowledge, this present study is the first to assess in vitro anticoccidial activity of the OL (*Olea europaea* L., var. Chemlal) extract and its phenolic compounds directly on the viability of *Eimeria* oocysts collected in broiler chickens.

It is known that the leaf is the main site of plant metabolism and can be considered as a potential source of bioactive compounds (Tsimidou and Papoti, 2010). Based on an oocyst wall alteration of *Eimeria*, this study showed that the OL extract (*Olea europaea*) had a destructive effect of sporulated oocysts. This anti-*Eimeria* activity may be attributed to bioactive molecules of OL extracts individually or a synergistic interaction of bioactive compounds. It would be more effective to control or treat coccidiosis by the use of a multiple compound, which requires a combination of immunostimulators that induce a good response of essential oils and other natural compounds from medicinal plant extracts that can destroy the Eimeria oocyst or interfere with the life cycle (Quiroz-Castañeda, 2018).

In the present study, the characterization by HPLC of the OL extract shows the presence of large concentrations of polyphenolic or biophenols (cynarosid/luteolin-7-O-glucoside; tyrosololeuropein quercetinisohamnetin; neobavaisoflavone 2, 3-dihydro-amentoflavone quercetin-3-O-rutinoside-chlorogenic acid, isorhamnetin 3-O-(6"-O-feruloyl)-glucoside), diligustilide quercetin-o-(o-galloyl)-hexoside) that can be used as natural anticoccidial products. Previous investigations have studied that the polyphenol content of olive leaves is richer in bioactive phenolic compounds than fruits and olive oil (Lalas et al., 2011). In addition, one other study has demonstrated the efficacy of plants and plant extracts against mixed or *Eimeria* individual infections (Udo and Abba, 2018). The results of the present study correspond also with those published previously, which showed that maslinic acid (2-α, 3-β-dihydroxiolean-12-en-28-oic acid), an active compound in the leaves and fruits of the olive tree (*Olea europaea*), has an anticoccidial effect against *E. tenella* oocysts and also increases the weight in chicks treated (De Pablos et al., 2010). In addition, the active compound of *Olea europea* has been shown to affect *Toxoplasma gondii* parasites, which may be by inhibiting the serine proteases of the protozoan protein, that is a mechanism necessary for the entry of tachyzoites into the cytoplasm of the host cell (Aladedunye et al., 2008). Therefore, this activity, as well as anti-inflammatory (Aladedunye et al., 2008) and antioxidant properties (Montilla et al., 2003), may be responsible for the anticoccidial properties. Moreover, it has been shown that the use of natural antioxidants such as polyphenols of olive by-products is safer than synthetic polyphenols in chicken diets in order to reduce lipid oxidation (Starćevic´ et al., 2015). Also, Varmaghany et al. (2013) have shown that an olive leaf supplementation diet (oleuropein content, 72.63 mg/g) has an anti-hypertensive effect and reduces the incidence of ascites without affecting the performance of broilers.

Khalafalla and Daugschies (2011) have demonstrated that curcumin, natural polyphenolic component derived from *Curcuma longa*, inhibited the cell invasions of *E. tenella* sporozoites in vitro and in vivo. Thus, anticoccidial activity of *M. oleifera*, including anti-inflammatory and antioxidant properties, was attributed to the biological constituents, including ascorbic acid, flavonoids, phenolics and carotenoid (Abdel-Latif et al., 2017). In other contexts, antimicrobial effects of phenolic compounds are targeted against the bacterial cell wall affecting the cell wall structure (Botta et al., 2005). This is supported by Molan et al. (2009) who explained that condensed tannins could penetrate the wall of the oocyst and cause damage to the cytoplasm by inactivating endogenous enzymes responsible for the sporulation process. Our results in vitro showed the low destruction rate of *Eimeria* oocysts that could be explained by the difference in oocyst resistance. This can be attributed to the impermeability of the inner phospholipid membrane to hydrophilic substances.

In the current study, the OL extract showed low activity with an LC_{50} of 192.94 mg/mL for a reduction rate of 19.4% compared with allicin hydraulic extract with an LC_{50} of 180 mg/mL for an inhibition percentage of 99.9% (Alnassan et al., 2015). In another study, the aqueous extract of *T. sanguine* containing phenolic components at a concentration of 2.5 mg/mL significantly inhibited ($P < 0.05$) the capacity of the sporozoites *E. tenella* and *E. necatrix* (Konan et al., 2012). It is important to underline that our observations regarding the concentration of the extraction solvent at 60% ethanol of the OL extract are divergent with those reported by Gadelhaq et al.(2018), where a significant effect of inhibition of sporulation and oocyst deterioration occurred at ethanolic concentrations of 50–70%. In this study, the low destruction rate of oocysts could be explained by the difference in oocyst resistance. This can be attributed to the presence of internal phospholipid membrane, known for its impermeability to lipophilic compounds. This dif-

ference between the reduction rates of oocysts can be attributed to the molecular structure, nature (lipophilic or hydrophilic), the mode of action of these synthetic phenolic compounds, molecular weight and the chemical composition of the oocyst wall. The anticoccidial potential effect of *A. annua* is generally known to have a multitude of antioxidants (vitamin A, C and E) including flavonoids such as quercetin (Bohorun et al., 2004). It has been confirmed that phenols interact with cytoplasmic membranes and change their cation permeability leading to impairment of crucial processes in coccidia cells and finally leading to their death (Sikkema et al., 1995). Our results are in agreement with those of Abuakkada and Ellakany (2008) who reported that diclazuril was more effective in decreasing the oocyst output of isolates of *E. tenella*.

Conclusion

The findings of the present study showed that it could be concluded that the OL (*Olea europaea* L., var. Chemlal) extract possesses the ability to destroy *Eimeria* spp. collected from naturally infected broiler chickens. In addition, quercetin and oleuropein were tested to evaluate separately in vitro anticoccidial activity against *Eimeria* oocysts for the first time. From this in vitro experiment, our observations showed that the phenolic compound of the OL extract tested separately possesses an anti-*Eimeria* effect. In future, in vivo investigations are required to assess the efficiency of the OL bioactive compounds in broiler chickens.

Conflict of interest

The authors declared that there is no conflict of interest.

Acknowledgement

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Authors' contributions

NID carried out the experimental work and wrote the manuscript. CN and MAA participated in biochemistry analysis of extract plant and reviewed the manuscript. AA designed, supervised the experimental study and reviewed the manuscript. All authors read and approved the final manuscript.

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