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In vitro Sporulation, Oocysticidal Sporulation Inhibition of *Eimeria papillate* and Cytotoxic Efficacy of Methanolic Extract of *Thymus daenensis* Leaves

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ABSTRACT

Background: *Thymus daenensis* is a phenol-rich species of the *Thymus* genus that possesses many biological and pharmaceutical activities.

Methods: This study used five groups of TDLE extract (50, 100, 200 and mg/mL). Distilled water and mebendazole (10 mg/ml) were used as negative and positive controls. Five worms of similar size were placed in each Petri plate, together with the extract test solution (20 mL). The present work aims to study the phytochemical profiling, evaluation of anticancer properties and *in vitro* oocysticidal activity of methanolic *T. daenensis* leaves extracts (TDLE).

Result: Infrared spectroscopy (FT-IR) of TDLE proved eleven different active classes of chemical compounds. The extract is characterized by higher phenolic contents (250.5±2.7 mg/g of gallic acid) and flavonoids (25±0.3 mg/g of quercetin). Also, TDLE showed moderate cytotoxicity against breast cancer cell lines (MCF-7) and lung cancer cell lines (A549) using MTT assay with LC_{50} attributed to 388.66±3.5 µg/ml and 354.33±2.5 µg/ml, respectively. The *in-vitro* study revealed that TDLE concentrations of 300 mg/ml caused significant suppression of *Eimeria. papillata* oocysts sporulation and increased percent of sporulation inhibition compared to the other commercial products which showed different levels of sporulation. Our findings indicate that TDLE has anticoccidial activity, which encourages the conduct of numerous *In vivo* investigations to find an effective treatment.

Key words: Anticoccidial, Cytotoxicity, *E. papillata*, *Thymus daenensis*.

INTRODUCTION

Eimeriosis is a severe parasitic illness caused by a protozoan parasite of the genus *Eimeria* that infects birds and domestic animals (Blake *et al*., 2015). *Eimeria* spp. characterized by homoxenous fecal-oral life cycles. It can infect and proliferate rapidly in their hosts' digestive systems. It causes diarrhea, impaired growth and, in some cases, death (Orengo *et al*., 2012). Also, infections can lead to susceptibility to infections with clostridiosis, a cause of severe necrotic enteritis (Moore, 2016). As a result, this illness results in significant economic losses worldwide (Chapman, 2014). These parasites are transmitted between hosts by the ingestion of food or water contaminated with oocysts. The oocyst contains a multi-layered cell wall making it highly resistant to environmental variables so its control is very difficult. As a result, inhibiting the sporulation process is an important endeavor in controlling these parasites.

Also, Infection with *E. papillata* causes significant damage to the intestinal mucosa, inflammation and disturbance in antioxidant/oxidant biomarker (Abdel-Tawab *et al*., 2020). As a result, it's an excellent model for studying eimeriosis (Dkhil, 2013).

Chemoprophylaxis, is one of the traditional strategies to control parasitic diseases, but it is expensive. Furthermore, long-term usage of anticoccidial medications resulted in drug resistance (El Banna *et al*., 2016). There are also concerns regarding food safety and public health as a result of medicine residues in animal products, which motivates researchers to seek safer alternatives (Wunderlich *et al*., 2014). Plant products may offer an alternative option for coccidial management when resistance has not yet emerged (Abbas *et al*., 2012), lowering farmer input costs and protecting animal welfare (Abu El Ezz *et al*., 2020). The plant compounds' modes of action include oocyst wall degradation, cytoplasm damage, ion loss with reduction of proton motive force and also production of oxidative stress, all of which inhibition of invasion and impair *Eimeria* spp.

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development (Abbas *et al*., 2012; Nazzaro *et al*., 2013). So, plant extract and essential oils could be used as safe and effective alternative strategies to the present antiparasitic medications (Anthony *et al*., 2005).

Cancer is the second biggest cause of death in the world population, according to official World Health Organization data, with 9.6 million people expected to die from the disease in 2018 (Organization, 2018). Lung cancer is the most common type of cancer and the leading cause of death among males, according to the most recent edition of the International Agency for Research on Cancer (IARC), published in September 2018. When it comes to women, breast cancer is the most commonly diagnosed cancer and is the main cause of cancer (Bray *et al*., 2018).

There are no extremely effective drugs to treat most cancers. There is a strong demand for novel medicines that are very effective, have little toxicity and have little impact on the environment. Novel natural compounds provide prospects for drug development innovation (Cai *et al*., 2004). More than two-thirds of the medications currently utilized as anticancer therapies are derived from plants. Cancer cell death and reduced proliferative signaling and proliferation are key features of anticancer therapeutic approaches (Amin *et al*., 2009).

Thymus genus is an aromatic herb belonging to the most important Labiatae family (Lamiaceae) that is distributed worldwide. Extracts of this plant have been shown to contain many bioactive compounds, including flavonoids, thymol, eugenol, carvacrol, saponins and phenols with strong therapeutic value (Saleem *et al*., 2022). These ingredients indicate that this plant has a variety of beneficial effects, including anti-inflammation, antioxidant, antibacterial (Dorman and Deans, 2000), anticoccidial (Jamroz *et al*., 2003), anthelmintic (Rasooli *et al*., 2006) and antifungal properties (Shi *et al*., 2016).

T. daenensis is a thymol-rich species of *Thymus* that grows to a height of 6-30 cm and has hastate leaves, multi-stems, pillow shrubs and wooden bases(Heydari *et al*., 2019). TDLE has been shown to have many pharmacological properties, including antimicrobial activities (Mohammadpour *et al*., 2011; Teimouri, 2012), immunomodulatory (Amirghofran *et al*., 2012), tyrosinase inhibition ability (Nouri and Esmaeilian, 2012) and hypolipidemic effects (Nazari *et al*., 2013). However, few studies have documented *T. decussatus* chemical profiles and its biological activities (Saleh *et al*., 2020).

To our knowledge, it is the first study demonstrated anticoccidial and cytotoxicity of *T. daenensis*. So, the present investigation was carried out to determine the phytochemical constituents of the extract, assess the biological activity of TDLE through *in vitro* cytotoxicity against Breast (MCF-7) and lung (A549) cancer cell lines and their effects on *E. papillata* oocyst sporulation and inhabitation.

MATERIALS AND METHODS

This experiment was carried out in the Zoology Department of the College of Sciences-King Saud University University from 10/2/2023 to 10/9/2023. *T. decussatus* leaves were gathered in Al Badiya - Tabuk, Saudi Arabia. A taxonomist from the Botany Department (King Saud University, Riyadh, Saudi Arabia) identified and certified the plant material in the herbarium. The procedure described by Manikandan *et al*. (2008) was used to prepare the 70% methanol extract of *T. decussatus* leaves, with the following modifications: electric blenders (Senses, MG-503T, Korea) were used to powder the air-dried leaves of *T. decussatus,* leaf powder of *T. decussatus* (100 g) was macerated in 70% methanol for 24 hours at 4°C, then percolated 5-7 times to ensure complete extraction. After filtering, methanol was separated from the extract using a vacuum evaporator with low pressure and a temperature of 50°C. Before being used, the crude extract was lyophilized and stored at -20°C. Plant extract chemical consistent were evaluated on the optical spectrometer NICOLET 6700 (Thermo Scientific, Waltham, USA) FT-IR spectroscopy utilizing the KBr pellet method with a range of 400-4000 cm^{-1} (Abu Hawsah *et al*., 2023).

Total phenolic content (TPC) in *TDLE* has been estimated according to method of 30. Briefly, to 100 L of leaf extract, 300 L of sodium carbonate solution (20%) and 100 L of the Folin-Ciocalteu reagent were added. The sample was then incubated at room temperature in the dark for 30 minutes. The wavelength was measured using a UV-Visible spectrophotometer (SHIMADZU, UV-1800). The total phenolic in the samples was determined using the following linear equation (y= 0.0021x+0.0021 with R^2=0.9995) based on a standard curve produced using various gallic acid concentrations (25-400 g/mL). The total phenolic content was expressed in milligrammes per gramme of dry weight.

Total flavonoid content (TFC) in *TDLE* has been estimated according to method 31. The same volume of a 2% $AlCl_3$ water solution was mixed with 0.5 mL of methanol extract after two hours at 25°C, the wavelength was measured to be 420 nm. The TFC was estimated using the equation (y=0.0172× + 0.0507 with R^2=0.995) and a calibration curve developed from various quercetin standard values (50-0400 g/mL). The estimated TFC was represented by quercetin (mg/g DW).

Breast (MCF-7) and lung (A549) cancer cell lines were grown in DMEM medium (Gibco, USA) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/ streptomycin (Gibco, USA). The cells were incubated at 37°C in an incubator with a humidified atmosphere of 5% CO^2. The cytotoxic activity of *TDLE* was determined using an MTT assay. In a nutshell, cells were plated in a 96-well culture plate at a density of 5×10^4 per ml and allow to grow for 24 hours. The cells were then treated with various amounts of *T. decussatus* extract (500, 250, 125, 62.5, 31, 125 and 15.625 g/mL) and doxorubicin as a positive control. After 48 hours, each well received 10 L of MTT solution (5 mg/mL in PBS), which was then incubated for another 4 hours. After that, the formazan product was solubilized by adding 100 L of acidified isopropanol to each well and shaking the plate for 10 minutes. The absorbance was measured at 570 nm using a microplate reader (BioTek, USA).

% Cell viability =

$$\text{Mean absorbance} = \left[\frac{\text{Treated cells}}{\text{Untreated cells}}\right] \times 100$$

Concentration of *T. decussatus* extract that caused 50% inhibition (LC_{50}) has been determined from the dose-response curve of cell viability percentage using OriginPro software.

The parasite utilized in the study was a laboratory strain of *E. papillate*. The unsporulated oocysts were collected from the faeces of infected mice and storage until used.

In vitro, sporulation of *E. papillate* oocysts was examined using various TDLE doses. We examined four doses (300, 200, 100 and 50 mg/ml)/5 ml potassium dichromate containing 1×10^5 oocysts in this assay. Positive control oocysts were treated with 5 ml 2.5% potassium dichromate ($K_2Cr_2O_7$), while untreated control oocysts were left with water. Additionally, 8.3 mg amprolium (Veterinary Agriculture Products Company [VAPCO], Jordan), 109 l Dettol TM, 25 l phenol and formalin (5%) were evaluated in triplicate. All petri dishes used for these treatments were incubated for 72 and 96 hours at temperatures ranging from 25 to 29 degrees Celsius and relative humidity of 80%. At the end of the incubation time, the oocysts were washed in distilled water, as described by Fatemi *et al*. (Fatemi *et al*., 2015). After that, the samples were maintained at 4 degrees Celsius. According to (Thagfan *et al*., 2020) method the sporulation % and sporulation inhibition percentage were recorded and counted with a haemocytometer.

$$\text{Sporulation (Sp)} = \frac{\text{Number of sporulation oocysts}}{\text{Total number of oocysts}} \times 100$$

Sporulation (Sp) inhibition percentage =

$$\frac{\text{Sp\% of control - Sp\% of extract}}{\text{Sp\% of control}} \times 100$$

One-way analysis of variance (ANOVA) was used to examine the data in SigmaPlot® version 11.0 (Systat Software, Inc., Chicago, IL, USA). Differences between groups were considered significant at a p-value≤0.01.

RESULTS AND DISCUSSION

Traditional plant-based medications have many potential advantages: They are inexpensive, widely available and there has been no evidence of resistance to whole-plant extracts, probably because to the synergistic action of many ingredients. Also, because there are multiple active compounds, each at a smaller dose than is required when a single drug is provided, phytotherapy may have less side effects than chemotherapy. Therefore, Screening should be tailored to detect a wide spectrum of plant extract activities (Willcox and Bodeker, 2000); (Ozioma and Chinwe, 2019). Herein, we investigate the biological activity of one of the most important Lamiaceae family, *T. decussatus*. In the present study, *TDLE* possessed a high phenolic content (250 mg of gallic acid/ g DW). Similarly, (Oubihi *et al*., 2020) reported a high phenolic concentration (243.08 mg/g gallic acid) in methanolic preparations of *Thymus leptobotrys* aerial parts. *Thymus transcaspicus* and *T. serpyllum*, on the other hand, exhibit modest phenolic content (varying from 22.14 to 37.62 mg gallic acid equivalents/g DW) (Sarfaraz *et al*., 2021). However, variations in phenolic concentration may be attributed to the use of different standards and extraction circumstances (Gharibi *et al*., 2013). FT-IR spectra of *T. decussatus* extract showed in (Fig 1, Table 1). Both of them illustrated the characteristic functional groups. band at 3418.07 cm^{-1}, confirming N-H stretching of aliphatic primary amine, 2926.26 cm^{-1}, implicit C-H stretching for the presence of alkane, C-H binding at 1651.88 cm^{-1}, emphasizes the presence of aromatic compound, band at 1539.11 cm^{-1} corresponds to N-H bending for the presence of amine, C-H binding at 1451.22 cm^{-1} confirmed the

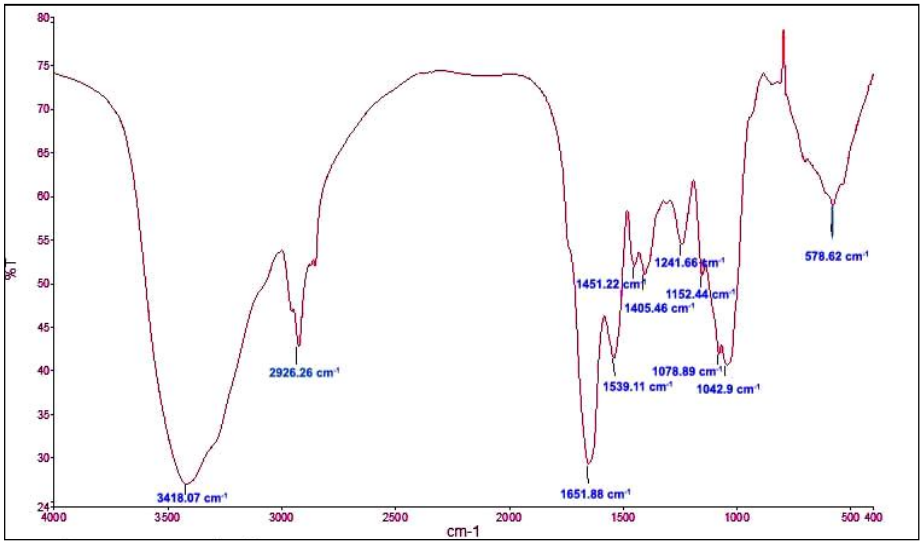


Fig 1: FTIR of TDLE displays the material's functional properties.

presence of alkene, The band at 1405.46 cm^{-1} coincides with S=O stretching for the presence of sulfate, three bands at 1241.66, 1152.44 and 1078.89 cm^{-1} corresponds to C-O stretching for the presence of aromatic ester, aliphatic ether and primary alcohol, respectively. Broad band at 1042.95 cm^{-1} (CO-O-CO stretching) assigned to anhydride, band at 578.62 cm^{-1} (C-I stretching) to halo compound.

Fig 2 showed the total flavonoids and phenolics content in the TDLE. The extract possesses high phenolic concentration (250.5±2.7 mg/g of gallic acid) and flavonoids (25±0.3 mg/g of quercetin). Flavonoid content was also demonstrated in the present study (25 mg quercetin/g DW). according to the previous report, total flavonoid content is highly varied among *Thymus* species. The TFC ranged from 1.77 to 8.72 mg QE/g DW was obtained from *T. trautvetteri* and *T. vulgaris* species, respectively (Sarfaraz *et al*., 2021). In contrast, high TFC was found in the methanolic extract of *Tymus transcaspicus* 179.28 mg/g RE (Oubihi *et al*., 2020). However, many plant species, have distinct strategies for distributing flavonoids across their subcellular compartments, such as the buildup of soluble carbohydrates and the balance between carbohydrate sources and sinks (Gharibi *et al*., 2016).

As observed in the MTT assay, TDLE decrease the viability of cell in concentration dependent manner, whereby the concentrations of 500 µg/ml showed toxicity against 63% the MCF-7 (Fig 3). Additionally, this compound was shown to be safe for normal cells up to a concentration of 300 µg/ml with LC_{50} attributed to 388.66±3.5 µg/ml. ASLE demonstrated cytotoxic effects on the A549cell line, at a high concentration of 400 and 500, causing cell death at a rate of 63, 53% and LC_{50} at 354.33±2.5 µg/ml. TDLE extracts have been examined for their antitumoral efficacy, underpins their potential as sources of chemotherapeutic drug sources. Our data demonstrated that TDLE decreased the viability of MCF-7 and A547 cell lines in a concentration-dependent manner. Previously, T. vulgaris aqueous decoction (IC_{50} values of 376.8 and > 500 g/mL, respectively) and hydroethanolic extracts (IC_{50} values of 442.45 and 254.25 g/mL, respectively) revealed low cytotoxic action against Caco-2 and HepG2 cells after 48 hours (Taghouti *et al*., 2020). Furthermore, *T. serpyllum* methanolic extracts decreased viability of two human breast cancer cell lines (MCF-7 and MDAMB-231) after 72 h exposure (IC_{50} of 509 and 276 µg/mL, respectively), proving the extract's anticancer property (Bozkurt *et al*., 2012). Furthermore, *T. serpyllum* and *T. vulgaris* extracts exhibit cytotoxicity on two

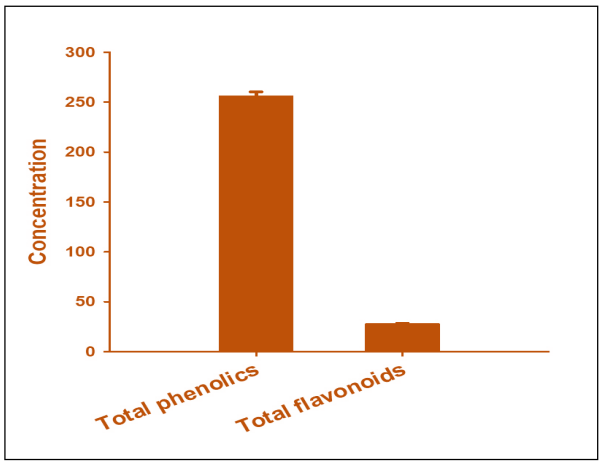


Fig 2: Total content of phenolics and flavonoids in the methanolic extract of the *T. decussatus* plant.

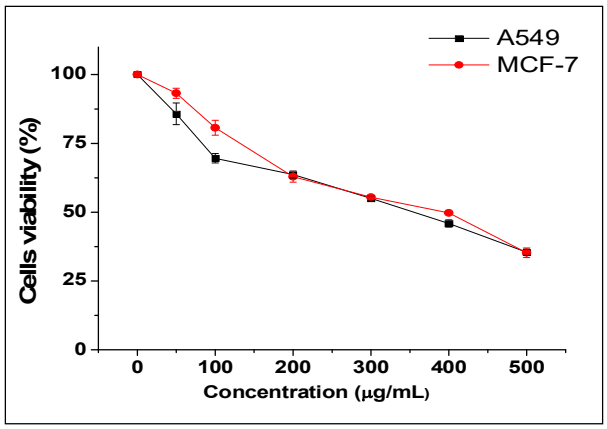


Fig 3: Cytotoxicity (MTT) assay for tested TDLE at different concentrations (µg/mL) against Lung (A549) and breast (MCF-7) cancer cell lines after 48 h of incubation.

Table 1: FT-IR for *Thymus daenensis* leaves extract.

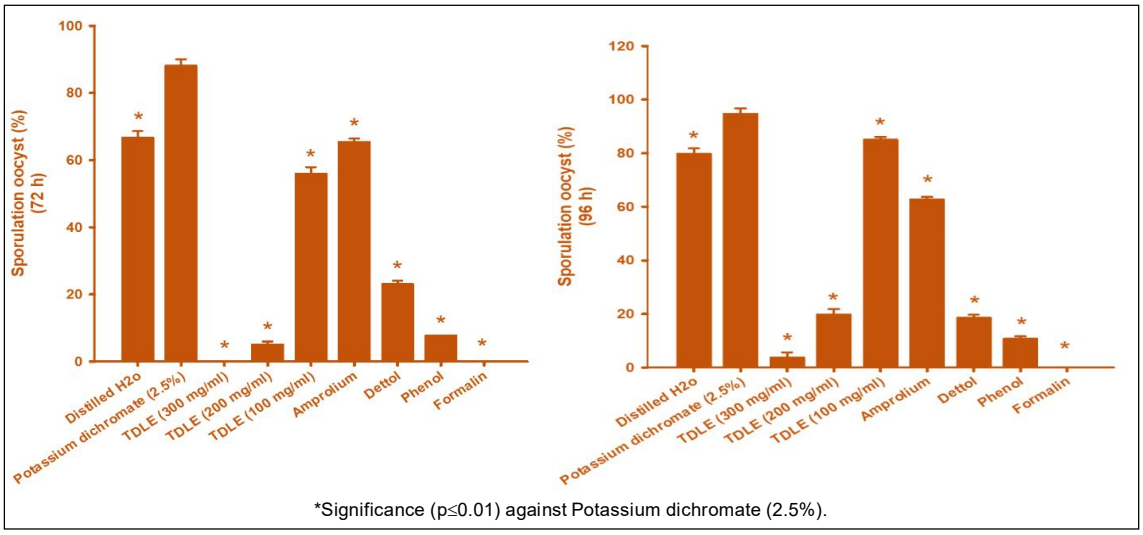
Absorption (cm^{-1})	Transmittance (%)	Appearance	Group	Compound class
3418.07	19.54684	Medium	N-H stretching	Aliphatic primary amine
2926.26	16.73434	Medium	C-H stretching	Alkene
1651.88	9.446569	Weak	C-H bending	Aromatic compound
1539.11	8.801674	Medium	N-H bending	Amine
1451.22	8.299059	Medium	C-H bending	Alkene
1405.46	8.037373	Strong	S=O stretching	Sulfate
1241.66	7.100653	Strong	C-O stretching	Aromatic ester
1152.44	6.590433	Strong	C-O stretching	Aliphatic ether
1078.89	6.169824	Strong	C-O stretching	Primary alcohol
1042.95	5.964295	Strong, Broad	CO-O-CO stretching	Anhydride
578.62	3.308941	Strong	C-I stretching	Halo compound

human breast cancer cell lines: Adriamycin-resistant MCF-7/Adr and wild-type MCF-7/wt at high concentrations only, with LC_{50} values of 399 and 409 µg/mL, respectively (Berdowska *et al*., 2013).

Thymus phenolic-rich extracts have been shown to exhibit anti-carcinogenic activity on cancer cell lines *via* a different mechanism of action. These include oxidative stress and cancer cell death (Satooka and Kubo, 2012), apoptotic cancer cell death (Deb *et al*., 2011) and antiproliferative efects on cancer cells (Mastelic *et al*., 2008). Contrary, antioxidant activity (Mapelli *et al*., 2016), protective effects (Hsu *et al*., 2011), anti-infammatory/immunomodulatory effects (Gholijani *et al*., 2015) and antigenotoxic effects (Slamenova *et al*., 2007) may be the key mechanisms of thymol's anti-carcinogenic activity in normal cells.

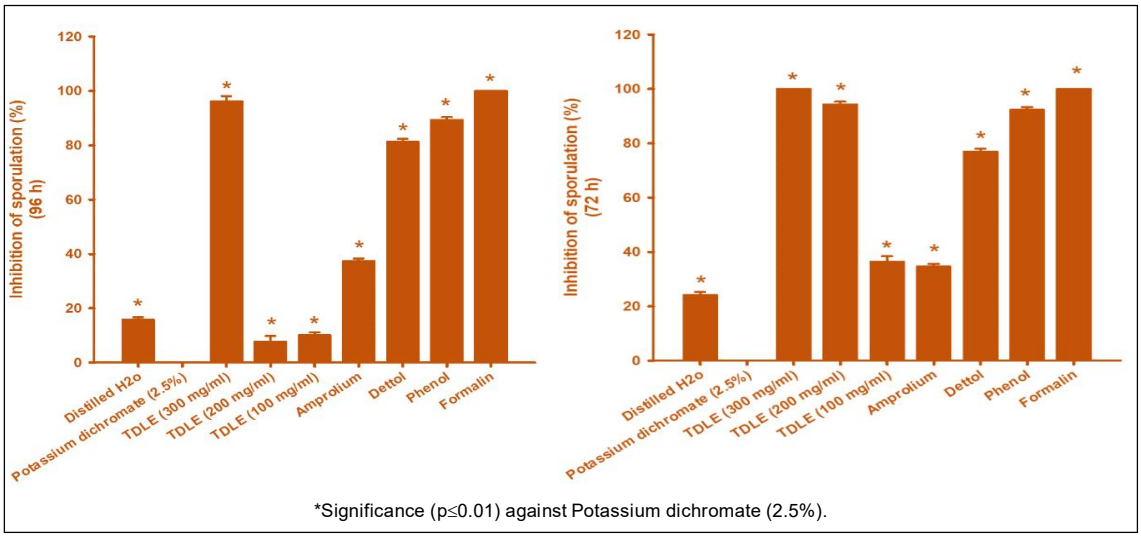
At 72 and 96 hours, the percentage of sporulated oocysts and inhibition were determined for the control and treatment groups. No sporulation was seen after 72 hours of incubation of *E. papillata* oocyst with doses of 300 mg/mL of TDLE. However, sporulation levels of 5% and 55.9% were observed at concentrations of 200 and 100 mg/mL, respectively. In comparison, the control group (2.5% potassium dichromate solution) demonstrated 88% sporulation. Sporulation% rose throughout 96 hours (Fig 4), with values of 2.7%, 19.8% and 85.05% at doses of 300, 200 and 100 mg/mL, respectively. Also, at 72 h, the rates of sporulation (%) in the DettolTM, phenol and formalin 5% were 23.08%, 7.7% and 0%, respectively (Fig 4) and at 96 h, they were 18.67%, 10.67% and 0%, respectively.

On the other hand, the highest sporulation inhibition (100%) was obtained for TDLE at a concentration of 300 mg in 72 h (Fig 5). While the levels of sporulation inhibition for amprolium, Dettol™, phenol and formalin 5% were 37. 33%, 81.33%, 89.33% and 100%, respectively, at 96 h (Fig 5), while, at 72 h it was 34.61%, 76.92%, 92.30% and 100% respectively.



*Significance (p≤0.01) against Potassium dichromate (2.5%).

Fig 4: Oocysticidal effects of TDLE on the oocyst sporulation percentage at 72 and 96 h.



*Significance (p≤0.01) against Potassium dichromate (2.5%).

Fig 5: Oocysticidal effects of TDLE on the Inhibition of oocyst sporulation (%) at 72 and 96 h.

Conacring the anticoccidial activity, TDLE possesses oocysticidal activity *in vitro* in a concentration-dependent manner, which is attributed to several bioactive phytochemical ingredients such as phenols flavonoids, thymol, eugenol, carvacrol, saponins and flavonoids which can interfere with the membrane permeability of pathogens, causing a cascade of reactions that involve the entire cell and eventually leads to its death (Nazzaro *et al*., 2013) and (Amarowicz *et al*., 2009).

Thyme extract containing thymol destroyed *E. tenella* oocysts (Abbas *et al*., 2012). *T. vulgaris* was found to be capable of destroying parasites oocysts and sporozoites (Muthamilselvan *et al*., 2016). Also, (Remmal *et al*., 2013; Remmal *et al*., 2011) observed lysis effect of thymol. In addition, (Giannenas *et al*., 2003) and (Küçükyilmaz *et al*., 2012) had discovered that oregano essential oils, which are high in thymol and carvacrol, help to improve animal health during a coccidia challenge and lower the number of oocysts shed in feces.

In a prior study, the efficacy of plant extracts as an alternative parasite treatment was determined by their anti-parasitic activity as well as their ability to boost the host immune system (Anthony *et al*., 2005). The current investigation showed complete inhibition of sporulation at 5% formalin which agrees with (Thagfan *et al*., 2020). On the contrary, (Kasem *et al*., 2019) reported a significant effect of formalin 10% on *E. tenella*. Sporulation. Formalin can react with the protein and inhabit the sporulation process *in vitro* (Fraenkel-Conrat *et al*., 1945).

Other used disinfectant as Dettol™ and Phenol inhibited sporulation by 81.33%, 89.33% respectively, which is consistent with (Mai *et al*., 2009) and (Gadelhaq *et al*., 2018) that reported that the oocyst wall is impermeable to water-soluble component and resistant to proteolysis.

CONCLUSION

This study revealed that *T. decussatus* is a rich source of phenolic and flavonoid consistent. It has anti-coccidial and antitumor properties. As a result, TDLE could be effective as an alternative product for controlling coccidiosis and developing antitumoral chemicals.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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