

# *In Vitro and In Silico* Anticoccidial Activity of *Opuntia ficus-indica* Seed Oil and Hexane Extract: Comprehensive GC-MS Profiling and Mechanistic Insights

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## Research Article

**Keywords:** Eimeria spp, Anticoccidial activity, Phytogetic compounds, Opuntia ficus-indica, in vitro, GC-MS, Molecular docking

**Posted Date:** July 25th, 2025

**DOI:** <https://doi.org/10.21203/rs.3.rs-7156619/v1>

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**Additional Declarations:** No competing interests reported.

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**Version of Record:** A version of this preprint was published at Acta Parasitologica on October 16th, 2025. See the published version at <https://doi.org/10.1007/s11686-025-01137-5>.

# Abstract

The anticoccidial potential of *Opuntia ficus-indica* seed oil (OFI-SO) and its hexane extract from seed press cake (OFI-HexPC) was evaluated against *Eimeria* spp. using integrated in vitro and in silico approaches.

Sporulated *Eimeria* oocysts were exposed to increasing concentrations (2–64 mg/mL) of OFI-SO and OFI-HexPC in Hank's balanced salt solution. Oocyst viability was determined by microscopic counting, while membrane integrity was assessed by quantifying the release of 273 nm-absorbing substances. Toltrazuril (25 mg/mL) was used as a positive control.

GC-MS profiling of both extracts revealed linoleic acid (54.2%) and oleic acid (25.7%) as the main bioactive fatty acids.

GC-MS profiling revealed that linoleic acid (69.63%) was the major fatty acid in OFI-SO, while oleic acid (44.20%) predominated in OFI-HexPC. Both OFI-SO and OFI-HexPC significantly reduced oocyst counts and induced a dose-dependent increase in DNA release.

In silico molecular docking was performed against three key *Eimeria* enzymes (EtDHODH, CDPK, and PKA). Identified fatty acids showed moderate binding affinities through hydrophobic interactions. Redocking validation (RMSD < 2 Å) confirmed the reliability of the docking protocol. ADMET predictions indicated favorable pharmacokinetics and safety profiles.

These findings suggest that OFI-SO and OFI-HexPC act via a dual mechanism involving membrane disruption and enzymatic inhibition, supporting their potential as natural alternatives to synthetic anticoccidials in poultry production.

## Introduction

Avian coccidiosis, an enteric parasitic disease caused by protozoa of the genus *Eimeria*, remains a major health and economic challenge in modern poultry production (Shirley et al., 2007; Chapman, 2014). Prevalent particularly in intensive farming systems, this parasitosis significantly compromises poultry growth, welfare, and productivity (Johnson, 1923; Taylor et al., 2022). Global economic losses associated with coccidiosis are estimated at approximately £10.4 billion annually (Blake et al., 2020), with recent assessments reporting losses of around £86.7 million per year in Algeria alone (Rahmani et al., 2024), underscoring its impact on national food security and farm profitability.

Chemoprophylaxis using synthetic anticoccidials remains the primary strategy for disease control in poultry (Taylor et al., 2022). However, the continuous use of these compounds has led to the emergence of drug-resistant *Eimeria* strains and raised serious concerns regarding drug residues in poultry products and potential risks to human health (Abbas et al., 2011; Chapman et al., 2013; Noack et al., 2019). These limitations have intensified the search for effective, safe, and sustainable alternatives (Abbas et al., 2012).

Phytogenic compounds derived from medicinal plants have gained considerable attention due to their diverse biological activities. Plant-based additives are known for their antimicrobial, antioxidant (Naidoo et al., 2008), antiviral (Bishop, 1995), antiparasitic, immunostimulant, and gut-modulating effects (Youn and Noh, 2001). Advances in phytochemical extraction and characterization techniques have reinforced the therapeutic value of many traditional medicinal plants (Gacem et al., 2020; Mahrose, 2021), supporting their use as alternatives to conventional antimicrobials in poultry production (Guèye, 1999).

*Opuntia ficus-indica*, a drought-tolerant species widespread in arid and semi-arid regions (Giraldo-Silva et al., 2023), is a rich source of lipophilic bioactive compounds, including fatty acids, sterols, and tocopherols. Recent studies suggest its potential antiparasitic effects, including anticoccidial properties (Amrane-Abider et al., 2023a, b).

The present study aims to investigate the anticoccidial potential of OFI-SO and OFI-HexPC against *Eimeria* spp. An integrated approach combining in vitro evaluation of oocyst integrity and in silico molecular docking of identified fatty acids targeting key *Eimeria* enzymes was adopted. This dual strategy aims to explore the therapeutic relevance of these natural products and support their development as promising alternatives to conventional anticoccidials in poultry health management.

## Material and Method

## Plant Material and Extraction of Non-Polar Compounds

*Opuntia ficus-indica* seed oil (OFI-SO) and the corresponding residual seed press cake were kindly provided by the NOPALTEC cooperative (Sidi-Fredj, Souk Ahras, Algeria), a local enterprise specialized in the processing of prickly pear-derived products. The plant material was harvested during the autumn season, corresponding to the full fruit maturity stage. Botanical identification was performed by the supplier's technical staff, including a certified botanist. Product quality and traceability were verified by their internal R&D department.

The oil was extracted by cold pressing of mature prickly pear seeds. The resulting seed press cake was air-dried, finely ground, sieved through a 1 mm mesh, and stored in amber glass bottles at room temperature until use. To isolate fatty acids and other non-polar constituents, OFI-HexPC was obtained by Soxhlet extraction using n-hexane at 100°C for 6 hours, following the protocol described by Kadda et al. (Kadda et al., 2023). The obtained extract was concentrated under reduced pressure using a Büchi rotary evaporator (Büchi, Switzerland), and subsequently stored at 4°C prior to analysis.

## Oocyst Collection and Identification

Oocysts of *Eimeria* spp. were isolated from fresh fecal samples collected from naturally infected broiler chickens in the Baghai region (Khenchela Province, eastern Algeria), following the procedure described by Carvalho et al. (Carvalho et al., 2011, 2025). The samples were processed for sporulation in a 2.5% potassium dichromate ( $K_2Cr_2O_7$ ) solution under optimal conditions of aeration, temperature, and humidity.

The concentration of sporulated oocysts (oocysts/mL) was determined using a *Malassez* counting chamber (Rahmani et al., 2021). For morphological identification, 100 sporulated oocysts were randomly selected and examined under a Leica microscope equipped with a digital camera. Image analysis was conducted using Motic Images Plus 2.0 software. Identification was based on morphometric parameters and compared with classical descriptions provided by Conway and McKenzie (Conway and McKenzie, 2007).

## Esterification and GC-MS Analysis

Fatty acids from OFI-SO and OFI-HexPC were converted into fatty acid methyl esters (FAMES) prior to gas chromatography–mass spectrometry (GC-MS) analysis. Briefly, 0.5 g of each sample was mixed with 5 mL of n-hexane, followed by the addition of 0.5 mL of 2 N KOH in methanol. After vortexing for 30 seconds, the mixture was centrifuged at 3000 rpm for 10 minutes. Then, 100 µL of the supernatant was collected and diluted with 1 mL of n-hexane. The resulting FAMES were stored at – 20°C until further analysis.

GC-MS analysis was performed using an Agilent 6890 Plus gas chromatograph coupled to an Agilent 5973 mass selective detector. The system was equipped with a splitless injector and an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; 5% phenyl–95% dimethylpolysiloxane). High-purity helium (N6) served as the carrier gas at a constant flow rate of 0.5 mL/min. The injector temperature was set to 250°C.

The oven temperature was programmed as follows: initial temperature of 70°C (held for 5 min), ramped at 10°C/min to 130°C (held for 2 min), followed by 3°C/min to 220°C (held for 4 min), and finally ramped at 10°C/min to 280°C (held for 5 min). The mass spectrometer operated in electron impact ionization mode with a quadrupole analyzer. Compound identification was carried out by comparing retention times and mass spectra to entries in the NIST and PubChem spectral libraries.

## Anticoccidial activity

The oocysticidal activity of OFI-SO and OFI-HexPC was evaluated in vitro according to the method described by Remmal et al (Remmal et al., 2011). Each extract was prepared in Hank's Balanced Salt Solution (HBSS) supplemented with 0.2% agar to ensure homogeneous oocyst dispersion and tested at six increasing concentrations (2 to 64 mg/mL). HBSS alone served as the negative control.

Each 1 mL reaction mixture contained 100  $\mu$ L of purified *Eimeria spp.* oocyst suspension ( $1.34 \times 10^5$  oocysts/mL for OFI-HexPC;  $2.2 \times 10^5$  oocysts/mL for OFI-SO), 700  $\mu$ L of phosphate-buffered saline (PBS), and 200  $\mu$ L of the test extract at the corresponding concentration.

After 24 hours of incubation at room temperature, oocyst destruction was estimated by microscopic counting using a Malassez chamber. Samples were then centrifuged (300  $\times$ g, 5 min, 4°C), and the absorbance of the supernatant was measured at 273 nm using a UV-Vis spectrophotometer (Cary 60, Agilent Technologies) to assess oocyst lysis.

Toltrazuril (Baycox 2.5%, 25 mg/mL, Bayer, Germany) was used as a positive control under the same test conditions. The lethal concentration ( $LC_{50}$ ) for each treatment was determined by regression curve analysis.

### Molecular Docking and ADMET Prediction

To analyze the interactions of the main compounds derived from OFI-SO and OFI-HexPC, as well as the positive control Toltrazuril and another anticoccidial drug, Diclazuril, with the active sites of three enzymes—calcium-dependent protein kinase (CDPK; PDB: 4YZB), cyclic AMP-dependent protein kinase (PKA; PDB: 4WB5), and *Eimeria tenella* dihydroorotate dehydrogenase (EtDHODH; PDB: 6AJE)—we performed molecular docking studies. Additionally, a comprehensive analysis of the ADMET properties (absorption, distribution, metabolism, excretion, and toxicity) of these compounds was carried out. The compounds examined include Toltrazuril, Diclazuril, linoleic acid, oleic acid, palmitic acid, and stearic acid.

The three-dimensional structures of the enzymes were obtained from the Protein Data Bank (PDB) (<https://www.rcsb.org/>). To ensure accuracy, ligands, ions, and water molecules were removed, and missing atoms in the crystallographic structures were added using Chimera (Pettersen et al., 2004). Active sites were identified by selecting amino acid residues within a 10 Å radius of the inhibitors. Energy minimization was performed with AutoDock Tools (Morris et al., 2009) to optimize structures and eliminate steric hindrance. The interactions between compounds and enzymes were modeled using Chimera and AutoDock Vina (Pettersen et al., 2004; Morris et al., 2009). Docking results were visualized using Discovery Studio Visualizer (Mohamed et al., 2023) to gain insights into molecular mechanisms.

To confirm the reliability of the docking protocol, redocking validation was performed (Adawara et al., 2022). The success of redocking was evaluated by calculating the root mean square deviation ( $RMSD < 2$  Å) (Aziz et al., 2023; Che et al., 2023) and analyzing docking scores, ensuring that the software could reproduce experimentally validated results,  $\Delta G$  is utilized to evaluate the binding affinity between ligands and receptors, with a negative  $\Delta G$  value indicating a favorable interaction.

Reliable online tools such as SwissADME, ProTox 3.0, and ADMETlab 2.0 (Daina et al., 2017; Ganorkar et al., 2020; Xiong et al., 2021; Ganorkar and Vander Heyden, 2022) were used to assess the ADMET profiles, pharmacodynamic properties, therapeutic potential, and toxicity of the compounds.

## Statistical analysis

All statistical analyses were performed using R software (R.4.4.3.; 2025). Data were initially subjected to analysis of variance (ANOVA) to assess the effects of extract types and concentrations on parasite count and absorbance. When ANOVA indicated significant differences ( $p < 0.05$ ), post hoc multiple comparisons were conducted. Pearson correlation analysis and dose-response analysis using linear regression were also performed. The lethal concentration ( $LC_{50}$ ) was defined as the concentration that reduced the initial number of sporulated oocysts by 50%.

## Results and Discussion

### Oocysts identification

Five *Eimeria* species were identified based on the morphological characteristics of sporulated oocysts: *E. acervulina* (39%), *E. tenella* (23%), *E. mitis* (15%), *E. maxima* (14%), and *E. brunetti* (9%). The predominance of *E. acervulina* and *E. tenella* aligns with previous findings reported in various regions of Algeria. Djemai et al. (Djemai et al., 2016) reported *E. acervulina* and *E. maxima*

as the most prevalent species in poultry farms in the Jijel region. In Sétif, *E. tenella* was the most frequently detected species (68%), followed by *E. mitis* (10%) and *E. acervulina* (14%) (Sabrina, 2021). A similar distribution pattern was described by Rahmani et al. (Rahmani et al., 2021), who recorded *E. acervulina* (35%), *E. tenella* (30%), *E. maxima* (15%), *E. brunetti* (12%), and *E. mitis* (8%).

### Phytochemical Characterization of OFI-SO and OFI-HexPC by GC–MS

GC–MS analysis of OFI-SO revealed that fatty acids constituted 91.33% of its total composition (Table 1; Fig. 1). Linoleic acid was the dominant component (69.63%), followed by palmitic (10.28%) and stearic (6.88%) acids. Several minor constituents were also detected, including palmitoleic (0.41%), gadoleic (0.47%), arachidic (0.90%), erucic (0.48%), behenic (0.49%), and lignoceric (0.34%) acids.

The fatty acid profile of OFI-HexPC differed considerably (Table 2; Fig. 2), with oleic acid being the predominant fatty acid (44.20%), followed by palmitic (19.02%) and stearic (10.15%) acids. Minor quantities of gadoleic (2.65%), arachidic (2.99%), and nonadecenoic (2.11%) acids were identified, alongside trace amounts of erucic, behenic, and lignoceric acids.

These findings are broadly consistent with previous analyses of Algerian *Opuntia ficus-indica* oils. Benattia et al. reported lower linoleic acid content (60.23%) and higher proportions of palmitic (14.2%) and oleic acids (13.35%) (Benattia et al., 2019). In contrast, Chafaa et al. found a higher linoleic acid level (74.24%) with a comparable stearic acid proportion (6.75%) (Chafaa et al., 2024). Abderrahmane et al. observed 62.63% linoleic acid and significantly more oleic acid (20.36%) (Bouaouich et al., 2023). Such variation is likely due to genetic background, environmental conditions, and extraction techniques.

Outside Algeria, comparable values have been reported. An Iraqi variety displayed slightly higher linoleic acid content (72.90%) and greater palmitic acid proportion (15.12%) (Alsaad et al., 2019). A recent study from Saudi Arabia showed a fatty acid composition similar to the present work, reinforcing the general lipid stability of *O. ficus-indica* across arid regions (Alqurashi et al., 2022). In Tunisian samples, linoleic acid was again the major constituent (60.69%), followed by oleic (16.41%), palmitic (12.72%), stearic (3.20%), and palmitoleic (0.75%) acids (BORCHANI, 2022).

In contrast, Moroccan OFI-HexPC was reported to contain only four main fatty acids: linoleic (37.21%), palmitic (29.9%), oleic (27.16%), and stearic (5.92%) (Kadda et al., 2023), highlighting the significant impact of regional and varietal factors on seed cake composition.

Both OFI-SO and OFI-HexPC share several key fatty acids, though their relative abundances differ. Linoleic acid, a polyunsaturated fatty acid (PUFA), is widely recognized for its hypocholesterolemic, anti-inflammatory, and skin-protective properties, supporting its broad industrial relevance (Ramadan and Mörsel, 2003; Kadda et al., 2023). Palmitic acid, a saturated fatty acid, is associated with increased LDL cholesterol (Djeghim et al., 2024), whereas stearic acid has a neutral or potentially beneficial impact on lipid metabolism (Tong et al., 2020). These biochemical characteristics, combined with the high content of unsaturated fatty acids, suggest that both extracts may hold nutritional, pharmaceutical, and anticoccidial potential.

Table 1  
Volatile compounds identified by GC-MS in OFI-SO

| peak    | Retention Time RT (min) | Area % | Library/ID*          | Molecules        | Formule      |
|---------|-------------------------|--------|----------------------|------------------|--------------|
| 1       | 24,53                   | 0,41   | Hexadecenoic acid    | Palmetoleic acid | C16 :1(9)    |
| 2       | 25,05                   | 10,28  | Hexadecanoic acid    | Palmetic acid    | C16 :0       |
| 3       | 30,2                    | 69,63  | Octadecadienoic acid | Linoleic acid    | C18 :2(9,12) |
| 4       | 30,97                   | 6,88   | Octadecanoic acid    | Stearic acid     | C18 :0       |
| 5       | 35,66                   | 0,47   | Eicosenoic acid      | Gadolic acid     | C20 :1(9)    |
| 6       | 36,46                   | 0,9    | Eicosanoic acid      | Arachidic acid   | C20 :0       |
| 7       | 41,1                    | 0,48   | 13-Docosenoic acid   | Erucic acid      | C22 :1(13)   |
| 8       | 41,62                   | 0,49   | Docosanoic acid      | Behenic acid     | C22 :0       |
| 9       | 47,27                   | 0,34   | Tetracosanoic acid   | Lignoceric acid  | C24 :0       |
| 10      | 54,32                   | 1,45   | Stigmastan 3.5diene  |                  |              |
| *NIST 2 |                         |        |                      |                  |              |

Table 2  
Volatile compounds identified by GC-MS in OFI-HexPC

| PEAK    | RT    | Area % | Library/ID*         | Molecules          | Formula    |
|---------|-------|--------|---------------------|--------------------|------------|
| 1       | 19,1  | 0,75   | Nonanoic acid       | Nonanoic acid      | C9 :0      |
| 2       | 20,23 | 0,02   | Dodecenoic acid     | Lauroleic acid     | C12 :1     |
| 3       | 23,47 | 0,08   | Tetradecenoic acid  | Myristoleic acid   | C14 :1     |
| 4       | 24,53 | 0,86   | Hexadecenoic acid   | Palmetoleic acid   | C16 :1(9)  |
| 5       | 25,05 | 14,86  | Hexadecanoic acid   | Palmetic acid      | C16 :0     |
| 6       | 28,1  | 0,2    | Tridecanoic acid    | Tridecanoic acid   | C13 :0     |
| 7       | 30,37 | 44,2   | Octadecenoic acid   | Oleic acid         | C18 :1     |
| 8       | 30,97 | 7,98   | Octadecanoic acid   | Stearic acid       | C18 :0     |
| 9       | 32,97 | 0,23   | Undecanoic acid     | Undecanoic acid    | C11 :0     |
| 10      | 34,76 | 0,68   | Carbonic acid       | Carbonic acid      | C1         |
| 11      | 35,66 | 2,65   | Eicosenoic acid     | Gadolic acid       | C20 :1(9)  |
| 12      | 36,46 | 2,99   | Eicosanoic acid     | Arachidic acid     | C20 :0     |
| 13      | 37,19 | 2,11   | Nonadecenoic acid   | Nonadecenoic acid  | C19 :1     |
| 14      | 41,1  | 0,55   | 13-Docosenoic acid  | Erucic acid        | C22 :1(13) |
| 15      | 41,62 | 1,21   | Docosanoic acid     | Behenic acid       | C22 :0     |
| 16      | 44,63 | 0,28   | Butanoic acid       | Butanoic acid      | C4         |
| 17      | 47,27 | 0,22   | Tetracosanoic acid  | Lignoceric acid    | C24 :0     |
| 18      | 49,52 | 0,11   | Pentacosanoic acid  | Pentacosanoic acid | C15 :0     |
| 19      | 54,32 | 0,11   | Stigmastan 3.5diene |                    |            |
| *NIST 2 |       |        |                     |                    |            |

## In Vitro Anticoccidial Activity of OFI-SO and OFI-HexPC

In vitro assays revealed a clear, concentration-dependent destructive effect on *Eimeria spp.* oocysts following treatment with OFI-SO, OFI-HexPC, and the synthetic anticoccidial agent Toltrazuril (Fig. 3). Both extract type and concentration had statistically significant effects on oocyst counts and the release of absorbent intracellular material ( $p < 0.001$ ), with a significant interaction between these factors ( $p < 0.001$ ; Tables 3 and 4). This dose-dependent effect was further confirmed by the progressive increase in absorbance at 273 nm (Fig. 4), indicative of membrane disruption and cytoplasmic leakage. No such effects were observed in the negative control group (HBSS).

Table 3  
Two-way ANOVA results for Parasite Numbers

| Source                                                                                                                        | df | SS     | MS     | F       | P       | Significance |
|-------------------------------------------------------------------------------------------------------------------------------|----|--------|--------|---------|---------|--------------|
| Extract Type                                                                                                                  | 2  | 901.45 | 450.72 | 3141.07 | < 0.001 | ***          |
| Concentration                                                                                                                 | 6  | 70.61  | 11.77  | 82.02   | < 0.001 | ***          |
| Extract Type: Concentration                                                                                                   | 12 | 49.80  | 4.15   | 28.92   | < 0.001 | ***          |
| Residuals                                                                                                                     | 42 | 6.03   | 0.14   |         | NA      |              |
| df: Degree of Freedom, SS, Sum Square, MS: Mean Square, * $p < 0.05$ , ** $p < 0.01$ , *** $p < 0.001$ , ns = not significant |    |        |        |         |         |              |

Table 4  
Two-way ANOVA results for Material Absorbance

| Source                                                                                                                        | Df | SS   | MS   | F      | p       | Significance |
|-------------------------------------------------------------------------------------------------------------------------------|----|------|------|--------|---------|--------------|
| Extract Type                                                                                                                  | 2  | 0.51 | 0.26 | 277.65 | < 0.001 | ***          |
| Concentration                                                                                                                 | 6  | 1.16 | 0.19 | 209.38 | < 0.001 | ***          |
| Extract Type: Concentration                                                                                                   | 12 | 0.55 | 0.05 | 50.05  | < 0.001 | ***          |
| Residuals                                                                                                                     | 42 | 0.04 | 0.00 |        | NA      |              |
| df: Degree of Freedom, SS, Sum Square, MS: Mean Square, * $p < 0.05$ , ** $p < 0.01$ , *** $p < 0.001$ , ns = not significant |    |      |      |        |         |              |

Post hoc comparisons demonstrated that OFI-HexPC consistently exhibited lower efficacy than both OFI-SO and Toltrazuril across all tested concentrations ( $p < 0.001$ ). By contrast, OFI-SO showed similar levels of efficacy to Toltrazuril ( $p = 1.000$ ), particularly at higher concentrations. Absorbance data confirmed these findings: while no significant differences were observed between treatments at low concentrations (0–4 mg/mL), OFI-HexPC's performance was significantly reduced at 8–64 mg/mL compared to OFI-SO and the positive control (Tables 5 and 6). A strong negative correlation between oocyst counts and absorbance (Table 7; Fig. 5) supports a mechanism involving membrane damage.

Table 5  
Tukey's HSD for Parasite Numbers across Extract Types at each Concentration

| Contrast                                  | Concentration | Estimate | SE   | Df | t.ratio | p.value |
|-------------------------------------------|---------------|----------|------|----|---------|---------|
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C1            | 11.20    | 0.31 | 42 | 36.21   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C1            | 11.20    | 0.31 | 42 | 36.21   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C1            | 0.00     | 0.31 | 42 | 0.00    | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C2            | 9.10     | 0.31 | 42 | 29.42   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C2            | 9.10     | 0.31 | 42 | 29.42   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C2            | 0.00     | 0.31 | 42 | 0.00    | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C3            | 9.10     | 0.31 | 42 | 29.42   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C3            | 9.10     | 0.31 | 42 | 29.42   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C3            | 0.00     | 0.31 | 42 | 0.00    | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C4            | 8.30     | 0.31 | 42 | 26.84   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C4            | 8.30     | 0.31 | 42 | 26.84   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C4            | 0.00     | 0.31 | 42 | 0.00    | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C5            | 7.30     | 0.31 | 42 | 23.60   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C5            | 7.30     | 0.31 | 42 | 23.60   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C5            | 0.00     | 0.31 | 42 | 0.00    | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C6            | 5.60     | 0.31 | 42 | 18.11   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C6            | 5.60     | 0.31 | 42 | 18.11   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C6            | 0.00     | 0.31 | 42 | 0.00    | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C7            | 5.57     | 0.31 | 42 | 18.02   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C7            | 5.57     | 0.31 | 42 | 18.00   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C7            | -0.01    | 0.31 | 42 | -0.02   | 1.000   |
| SE: Standard Error, df: Degree of Freedom |               |          |      |    |         |         |

Table 6  
Tukey's HSD for Material Absorbance across Extract Types at each Concentration

| Contrast                                  | Concentration | Estimate | SE    | Df | t.ratio | p.value |
|-------------------------------------------|---------------|----------|-------|----|---------|---------|
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C1            | 0.00     | 0.025 | 42 | 0.00    | 1.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C1            | 0.00     | 0.025 | 42 | 0.00    | 1.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C1            | 0.00     | 0.025 | 42 | 0.00    | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C2            | -0.03    | 0.025 | 42 | -1.21   | 0.700   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C2            | -0.04    | 0.025 | 42 | -1.41   | 0.497   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C2            | -0.01    | 0.025 | 42 | -0.20   | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C3            | -0.02    | 0.025 | 42 | -0.76   | 1.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C3            | -0.04    | 0.025 | 42 | -1.77   | 0.252   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C3            | -0.03    | 0.025 | 42 | -1.01   | 0.958   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C4            | 0.25     | 0.025 | 42 | 10.08   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C4            | 0.22     | 0.025 | 42 | 9.07    | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C4            | -0.03    | 0.025 | 42 | -1.01   | 0.958   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C5            | 0.27     | 0.025 | 42 | 10.89   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C5            | 0.25     | 0.025 | 42 | 10.08   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C5            | -0.02    | 0.025 | 42 | -0.81   | 1.000   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C6            | 0.48     | 0.025 | 42 | 19.15   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C6            | 0.43     | 0.025 | 42 | 17.13   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C6            | -0.05    | 0.025 | 42 | -2.02   | 0.151   |
| EXTRACT 1(OFI-HexPC) - EXTRACT 2(OFI-SO)  | C7            | 0.47     | 0.025 | 42 | 19.08   | 0.000   |
| EXTRACT 1(OFI-HexPC) - POSITIVE CONTROL   | C7            | 0.42     | 0.025 | 42 | 16.93   | 0.000   |
| EXTRACT 2(OFI-SO) - POSITIVE CONTROL      | C7            | -0.05    | 0.025 | 42 | -2.15   | 0.112   |
| SE: Standard Error, df: Degree of Freedom |               |          |       |    |         |         |

Table 7  
Pearson correlation between Parasite Numbers and Material Absorbance by Extract Type

| Extract Type                                   | Pearson_r | t_value | df     | p_value | CI_lower | CI_upper |
|------------------------------------------------|-----------|---------|--------|---------|----------|----------|
| EXTRACT 1(OFI-HexPC)                           | -0.923    | -10.493 | 19.000 | 0.000   | -0.969   | -0.818   |
| EXTRACT 2(OFI-SO)                              | -0.804    | -5.903  | 19.000 | 0.000   | -0.918   | -0.571   |
| POSITIVE CONTROL                               | -0.890    | -8.515  | 19.000 | 0.000   | -0.955   | -0.745   |
| df: Degree of Freedom, CI: Confidence Interval |           |         |        |         |          |          |

The dose-response curves (Figs. 6 and 7) indicated that all tested compounds exhibited concentration-dependent effects on both parameters. Toltrazuril demonstrated the highest potency with an  $LC_{50}$  of 47.00 mg/mL. Among the plant-derived compounds, OFI-SO showed slightly higher activity ( $LC_{50}$  = 47.05 mg/mL) compared to OFI-HexPC ( $LC_{50}$  = 50.09 mg/mL), confirming the superior activity of OFI-SO among the plant-derived treatments.

These results align with prior investigations on *Opuntia ficus-indica* extracts, where peel and flower fractions demonstrated lower efficacy ( $LC_{50}$  = 60.53 mg/mL and 66.04 mg/mL, respectively) (Amrane-Abider et al., 2023a, b). By comparison, Pistacia lentiscus oil showed a substantially lower  $LC_{50}$  (5.86 mg/mL), suggesting higher antiparasitic potency (Rahmani et al., 2021). Other plant-based products, such as olive leaf extracts, yielded varied results, with  $LC_{50}$  values ranging from 14.88 to 194.92 mg/mL depending on the bioactive component (Debbou-louknane et al., 2021). Variability in experimental protocols, Eimeria species, and extract composition likely account for these differences.

The potent activity observed for OFI-SO and OFI-HexPC is likely due to their high content of unsaturated fatty acids, particularly linoleic and oleic acids, as confirmed by GC–MS. These compounds are known to exhibit anti-protozoal activity, often through disruption of parasite membrane integrity and interference with ion transport (Ismaeil et al., 2025; Liu et al., 2025). The increased absorbance at 273 nm in treated groups suggests leakage of nucleotides and aromatic amino acids—typical indicators of cell lysis (Remmal et al., 2011).

These findings are further supported by similar effects reported in other parasitic systems. PUFAs such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) have demonstrated inhibitory activity against *Plasmodium falciparum*, disrupting schizont development and inhibiting nucleic acid synthesis (Kumaratilake et al., 1992; Krugliak et al., 1995). In helminths like *Schistosoma mansoni*, AA induced tegumental disintegration through sphingomyelinase activation, resulting in exposure of parasitic antigens (El Ridi et al., 2012). Comparable membrane-disruptive effects were also noted in oocysts exposed to plant extracts, leading to loss of cytoplasmic mass and parasite death (Abbas et al., 2019).

Taken together, these data suggest that OFI-SO and OFI-HexPC exert their anticoccidial effects through a combination of membrane disruption and intracellular destabilization. These preliminary findings justify further mechanistic investigation through molecular docking and in vivo validation.

### Molecular Docking Analysis

To elucidate the molecular mechanisms underlying the anticoccidial activity observed in vitro, molecular docking simulations were conducted on three key enzymes: dihydroorotate dehydrogenase (EtDHODH; PDB: 6AJE), calcium-dependent protein kinase (CDPK; PDB: 4YZB), and cAMP-dependent protein kinase A (PKA; PDB: 4WB5). These targets are critical to pyrimidine biosynthesis, intracellular signaling, and host cell invasion, and are widely recognized as relevant targets for anticoccidial drug discovery (Haste et al., 2012; Sato et al., 2020; Aljedaie et al., 2021).

The docking protocol was validated through redocking of the native ligands into their respective binding sites. The resulting RMSD values—1.018 Å for 4YZB, 0.861 Å for 4WB5, and 1.086 Å for 6AJE—demonstrated excellent overlap with crystallographic poses, confirming the reliability of the docking simulations.

Docking results (Figs. 8–11) showed that Diclazuril exhibited the strongest binding affinities, particularly with CDPK (−8.2 kcal/mol), followed by Toltrazuril (−7.7 kcal/mol). Diclazuril formed multiple hydrogen bonds (e.g., TYR339, SER340, TYR98) and hydrophobic interactions (e.g., ILE342, LEU70, LEU80). Toltrazuril displayed strong interactions through hydrogen bonds (GLY317, SER287, THR95), hydrophobic contacts (CYS111, LEU148, LYS50), and halogen bonding, which may enhance its target binding stability.

Among the fatty acids identified in OFI-SO and OFI-HexPC, linoleic, oleic, palmitic, and stearic acids displayed moderate binding energies (−5.4 to −6.5 kcal/mol). These values are consistent with other phytochemicals such as apigenin (−6.44 kcal/mol) and artemisinin (−5.83 kcal/mol) (Aljedaie et al., 2021). The binding modes were predominantly stabilized by hydrophobic interactions within the active sites, suggesting a potential for partial enzymatic inhibition.

Although the fatty acids exhibited weaker binding affinities than synthetic anticoccidials, their interaction profiles support a plausible mechanism of enzymatic interference, complementing the membrane-disruptive effects observed in vitro. In particular, linoleic acid demonstrated consistent binding behavior across all three targets and may significantly contribute to the dual anticoccidial action of OFI-SO and OFI-HexPC.

These findings support the hypothesis that the anticoccidial effects of *Opuntia ficus-indica* seed-derived extracts arise from a synergistic mechanism involving both structural damage to oocyst membranes and targeted inhibition of key parasite enzymes.

### **Analysis of ADMET Properties**

The ADMET analysis (Table 8) highlights that Diclazuril and Toltrazuril, used as reference anticoccidial drugs, display favorable pharmacokinetic properties but also present notable toxicological risks. Toltrazuril shows high respiratory toxicity and hormonal receptor interaction, while Diclazuril exhibits marked neurotoxicity, carcinogenic potential, and thyroid receptor binding. These toxic effects raise concerns about their safety for prolonged use in animals.

In contrast, the fatty acids evaluated in this study, linoleic, oleic, palmitic, and stearic acids, derived from OFI-SO and OFI-HexPC, demonstrate a favorable ADMET profile. They do not show hepatotoxicity, neurotoxicity, respiratory toxicity, carcinogenic activity, or cytochrome P450 inhibition. While only palmitic acid crosses the blood-brain barrier, all compounds display acceptable bioavailability and pharmacokinetic behavior. In addition to their low toxicity, these fatty acids showed notable biological activity in molecular docking analyses, reinforcing their potential as natural, safer anticoccidial agents. Their ability to target specific molecular pathways, combined with good tolerability, supports their inclusion in the development of alternative therapeutic formulations.

Table 8  
Analysis of ADMET Properties and Toxicity Comparison: Toltrazuril, Diclazuril, and Fatty Acids

|                            | Molecule             | Toltrazuril     | Diclazuril      | Linoleic Acid   | Oleic Acid      | Palmitic Acid   | Stearic Acid    |
|----------------------------|----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Physicochemical Properties | MW                   | 425.38          | 346.38          | 280.45          | 282.46          | 256.42          | 284.48          |
|                            | Csp3                 | 0.17            | 0.2             | 0.72            | 0.83            | 0.94            | 0.94            |
|                            | NRB                  | 5               | 3               | 14              | 15              | 14              | 16              |
|                            | NHA                  | 7               | 4               | 2               | 2               | 2               | 2               |
|                            | NHD                  | 1               | 1               | 1               | 1               | 1               | 1               |
|                            | TPSA                 | 111.39          | 91.54           | 37.3            | 37.3            | 37.3            | 37.3            |
| Lipophilicity              | Log P                | 3.64            | 2.75            | 2.75            | 4.57            | 4.19            | 4.67            |
| Water Solubility           | Log S                | Moderately      | Moderately      | Moderately      | Moderately      | Moderately      | Moderately      |
| Pharmacokinetics           | HIA                  | High            | High            | High            | High            | High            | High            |
|                            | BBB                  | No              | No              | No              | No              | Yes             | No              |
| Drug-likeness              | Lipinski             | 0               | 0               | 0               | 1               | 1               | 1               |
|                            | Ghose                | 0               | 0               | 0               | 1               | 0               | 1               |
|                            | Veber                | 0               | 0               | 0               | 1               | 1               | 1               |
|                            | Egan                 | 0               | 0               | 0               | 1               | 0               | 1               |
|                            | Muegge               | 0               | 0               | 0               | 1               | 1               | 2               |
| Medicinal Chemistry        | PAINS                | 0               | 0               | 0               | 0               | 0               | 0               |
| Organ toxicity             | Hepatotoxicity       | Active (0.67)   | Active (0.53)   | Inactive (0.55) | Inactive (0.55) | Inactive (0.52) | Inactive (0.52) |
|                            | Neurotoxicity        | Active (0.73)   | Active (0.88)   | Inactive (0.91) | Inactive (0.91) | Inactive (0.92) | Inactive (0.92) |
|                            | Respiratory toxicity | Active (0.75)   | Active (0.56)   | Inactive (0.84) | Inactive (0.84) | Inactive (0.85) | Inactive (0.85) |
| Toxicity end points        | Carcinogenicity      | Active (0.5)    | Active (0.5)    | Inactive (0.64) | Inactive (0.64) | Inactive (0.63) | Inactive (0.63) |
|                            | THRβ                 | Inactive (0.61) | Active (0.66)   | Inactive (0.91) | Inactive (0.78) | Inactive (0.78) | Inactive (0.9)  |
|                            | Clinical toxicity    | Active (0.61)   | Active (0.58)   | Inactive (0.61) | Inactive (0.61) | Inactive (0.64) | Inactive (0.64) |
| Metabolism                 | CYP1A2               | Inactive (0.69) | Inactive (0.64) | Inactive (0.91) | Inactive (0.91) | Inactive (0.84) | Inactive (0.84) |
|                            | CYP2C19              | Inactive (0.78) | Inactive (0.9)  | Inactive (0.98) | Inactive (0.98) | Inactive (0.98) | Inactive (0.98) |
|                            | CYP2C9               | Inactive (0.57) | Inactive (0.65) | Inactive (0.69) | Inactive (0.69) | Inactive (0.71) | Inactive (0.71) |

**MW** =Molecular Weight below 500, **NHA**=Num. H-bond Acceptors, **NHD**=Num. H-bond donors, **NRB**=Num. rotatable bonds, **Fsp3** =Fraction Csp3 acceptors, **HIA**= Human Intestinal Absorption, **CYP**= Cytochrome P450, **BBB** = Blood-Brain Barrier, **PAINS**=Pan Assay Interference compounds or frequent hitters or promiscuous compounds, **Log P**: logarithm of the compound partition coefficient between n-octanol and water (- 2 to 5), **TPSA**= Polar surface area (0-140), **THRβ** =Thyroid hormone receptor bet

|  | Molecule | Toltrazuril     | Diclazuril      | Linoleic Acid   | Oleic Acid      | Palmitic Acid   | Stearic Acid    |
|--|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|  | CYP2D6   | Inactive (0.8)  | Inactive (0.86) | Inactive (0.88) | Inactive (0.88) | Inactive (0.88) | Inactive (0.88) |
|  | CYP3A4   | Inactive (0.58) | Inactive (0.88) | Inactive (1.0)  | Inactive (1.0)  | Inactive (1.0)  | Inactive (1.0)  |
|  | CYP2E1   | Inactive (0.99) | Inactive (1.0)  | Inactive (0.99) | Inactive (0.99) | Inactive (0.99) | Inactive (0.99) |

**MW** =Molecular Weight below 500, **NHA**=Num. H-bond Acceptors, **NHD**=Num. H-bond donors, **NRB**=Num. rotatable bonds, **Fsp3** =Fraction Csp3 acceptors, **HIA**= Human Intestinal Absorption, **CYP**= Cytochrome P450, **BBB** = Blood–Brain Barrier, **PAINS**=Pan Assay Interference compounds or frequent hitters or promiscuous compounds, **Log P**: logarithm of the compound partition coefficient between n-octanol and water (– 2 to 5), **TPSA**= Polar surface area (0–140), **THRβ** =Thyroid hormone receptor bet

## Conclusion

This study provides compelling evidence for the anticoccidial efficacy of OFI-SO and OFI-HexPC, based on integrated in vitro and in silico investigations. OFI-SO demonstrated a pronounced oocysticidal effect, comparable to that of the synthetic anticoccidial Toltrazuril. This activity is likely attributable to its high content of bioactive unsaturated fatty acids, particularly linoleic and oleic acids.

Molecular docking analyses confirmed that these compounds are capable of interacting with key *Eimeria* enzymes, notably EtDHODH, CDPK, and PKA, suggesting a dual mode of action involving both membrane disruption and enzymatic inhibition. This mechanistic synergy enhances their potential as effective antiparasitic agents.

Furthermore, ADMET predictions revealed favorable pharmacokinetic and safety profiles for the tested fatty acids, contrasting with the organ-specific toxicities commonly associated with synthetic anticoccidials. These data support the candidacy of OFI-SO and OFI-HexPC as natural, safer alternatives to conventional chemoprophylactic agents in poultry production.

Future studies should focus on in vivo validation, dosage optimization, and large-scale application under commercial farming conditions to fully establish the practical utility of these phytogenic extracts.

## Declarations

### Ethics approval and consent to participate:

All authors approved.

#### Consent for publication:

All authors agree to the publication.

#### Conflicts of Interest:

The authors declare no conflicts of interest.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Funding:

This research was not funded

## Author Contribution

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

## Acknowledgement

The authors would like to express their gratitude to the Ministry of Higher Education and Scientific Research and the General Directorate of Scientific Research and Technological Development (DGRSDT) for their support. The authors also gratefully acknowledge the NOPALTEC cooperative (Souk Ahras, Algeria) for providing the plant material and technical assistance. Special thanks are extended to the staff of the Parasitology Laboratory at the Biotechnology Research Center (C.R.Bt) for their valuable support in the collection and morphological identification of *Eimeria* spp. oocysts. We also acknowledge the Environmental Laboratory, represented by Dr. Cherb Nora, for her collaboration and technical contributions during the experimental procedures.

## Data availability statement:

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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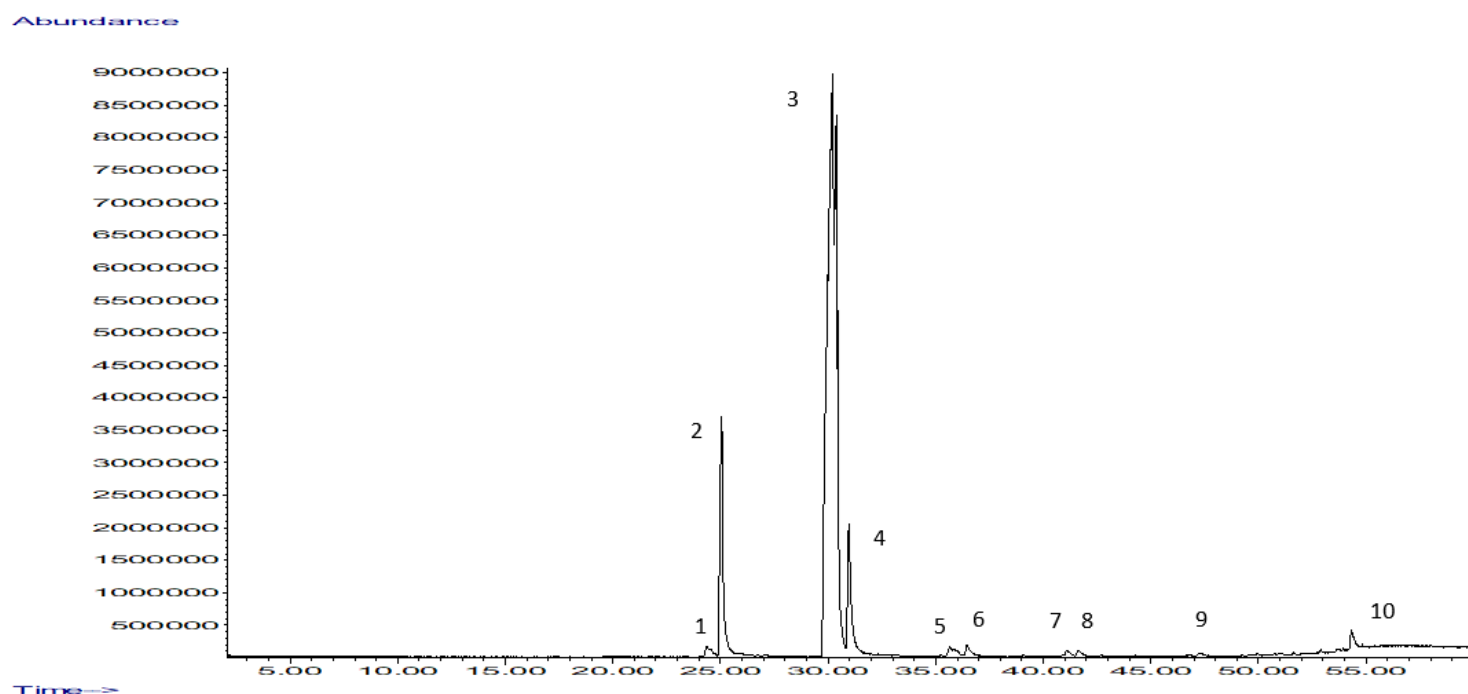
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## Figures



**Figure 1**

Chromatogram of GC-MS analysis of OFI-SO

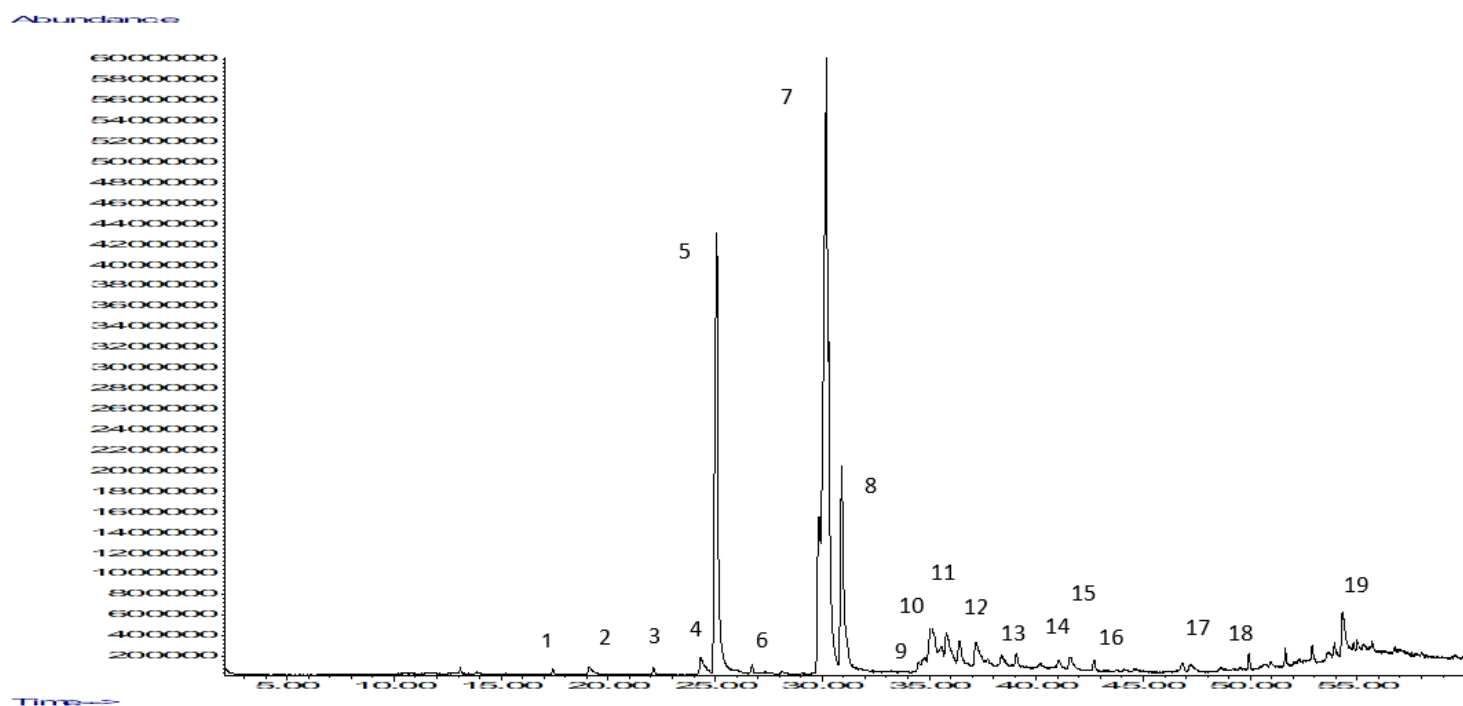


Figure 2

Chromatogram of GC-MS analysis of OFI-HexPC

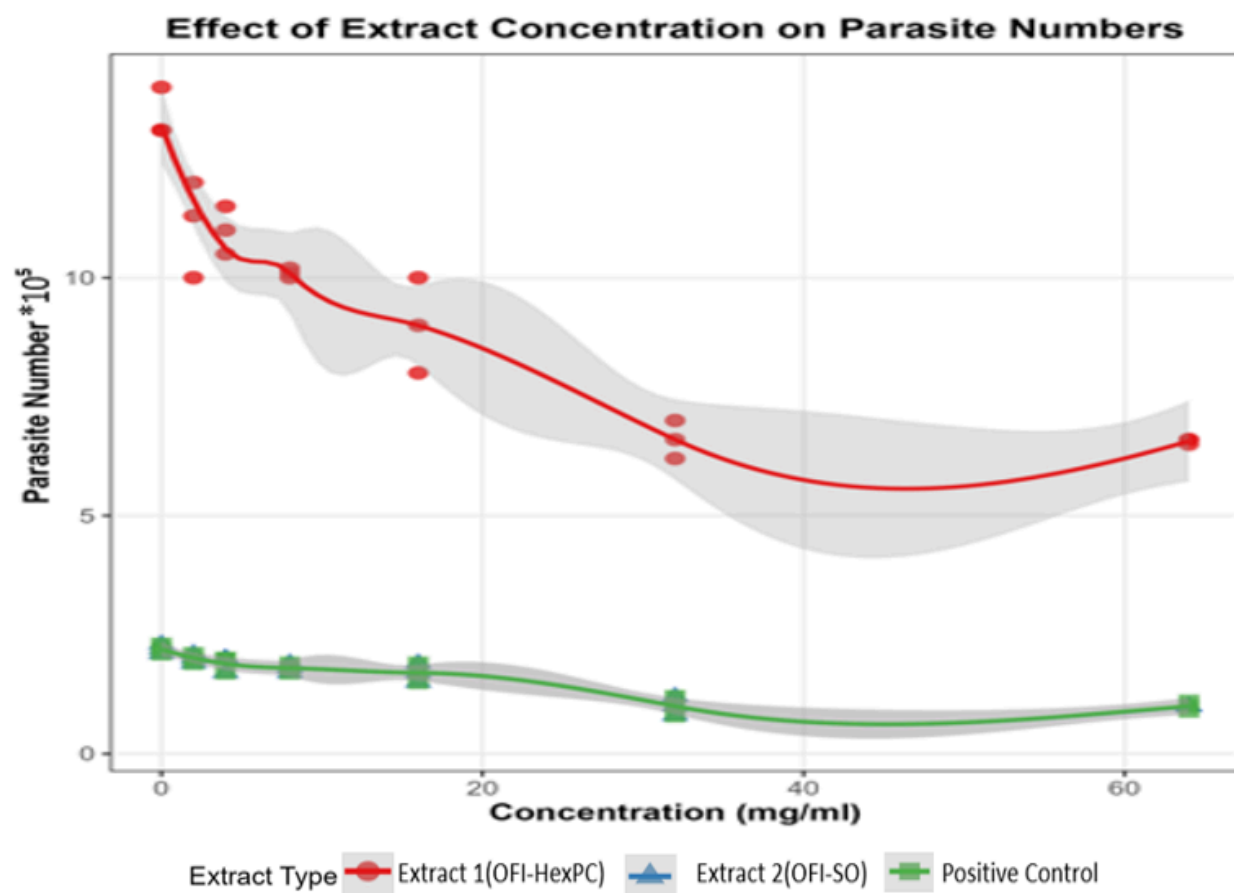


Figure 3

Scatter plot of parasite numbers vs. concentration with trend lines showing the dose-response relationship for each extract type

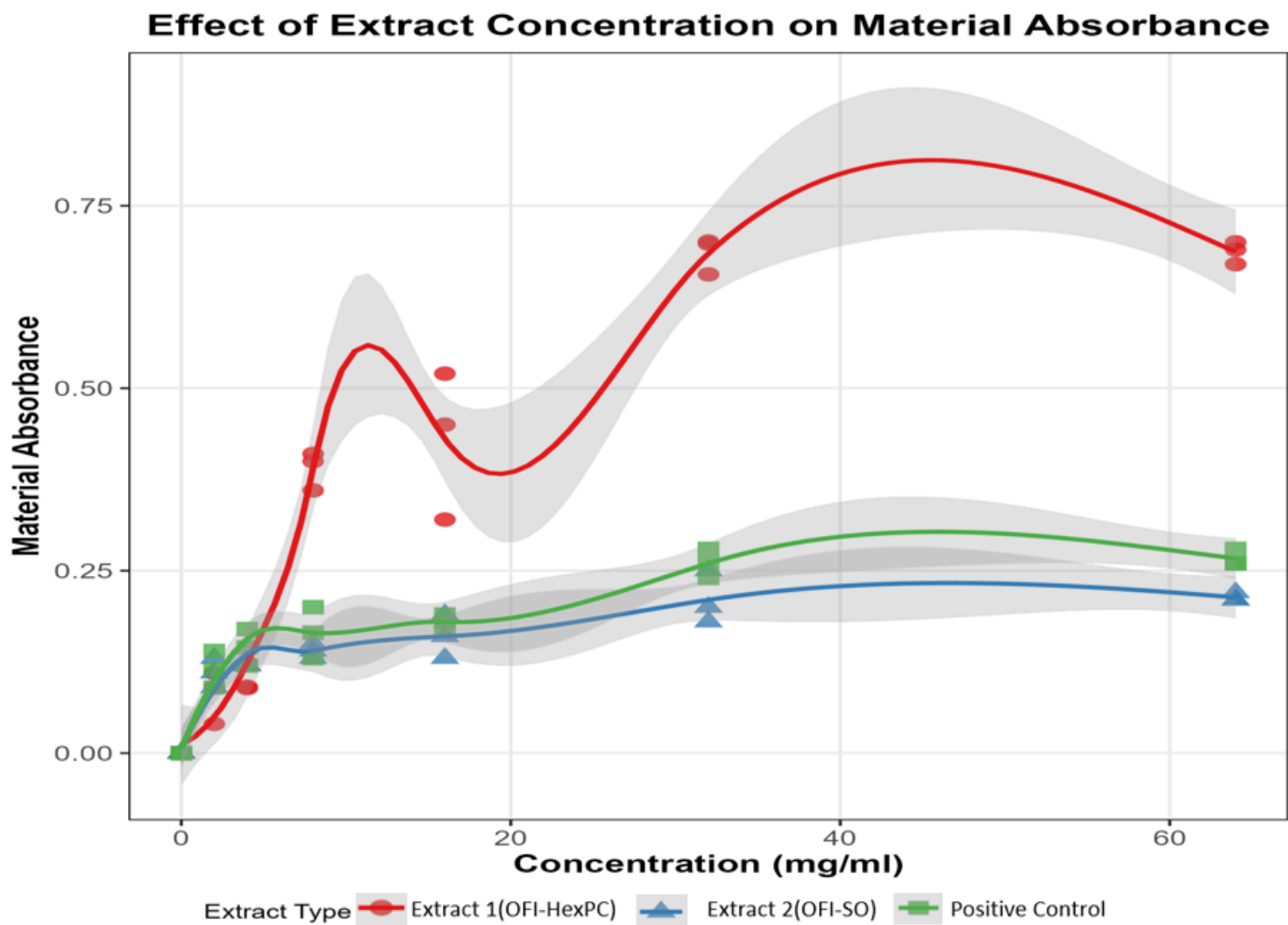


Figure 4

Scatter plot of material absorbance vs. concentration with trend lines showing the dose-response for each extract

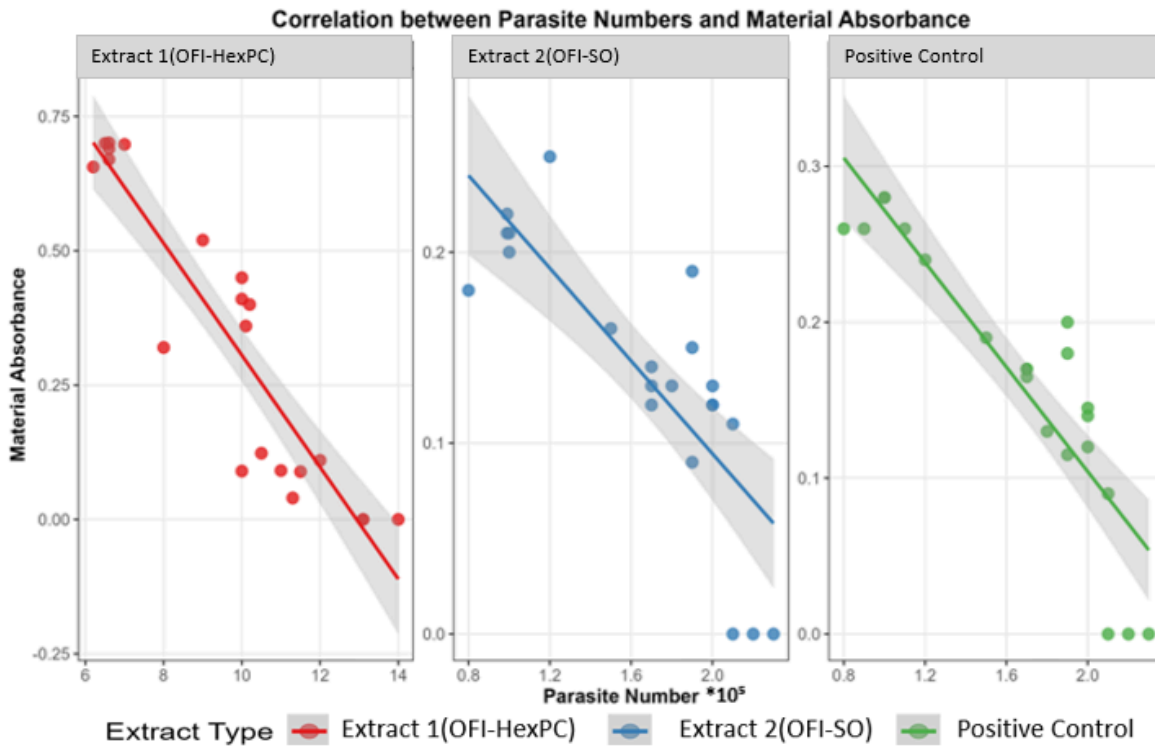


Figure 5

Scatter plots showing the correlation between parasite numbers and material absorbance for each extract type

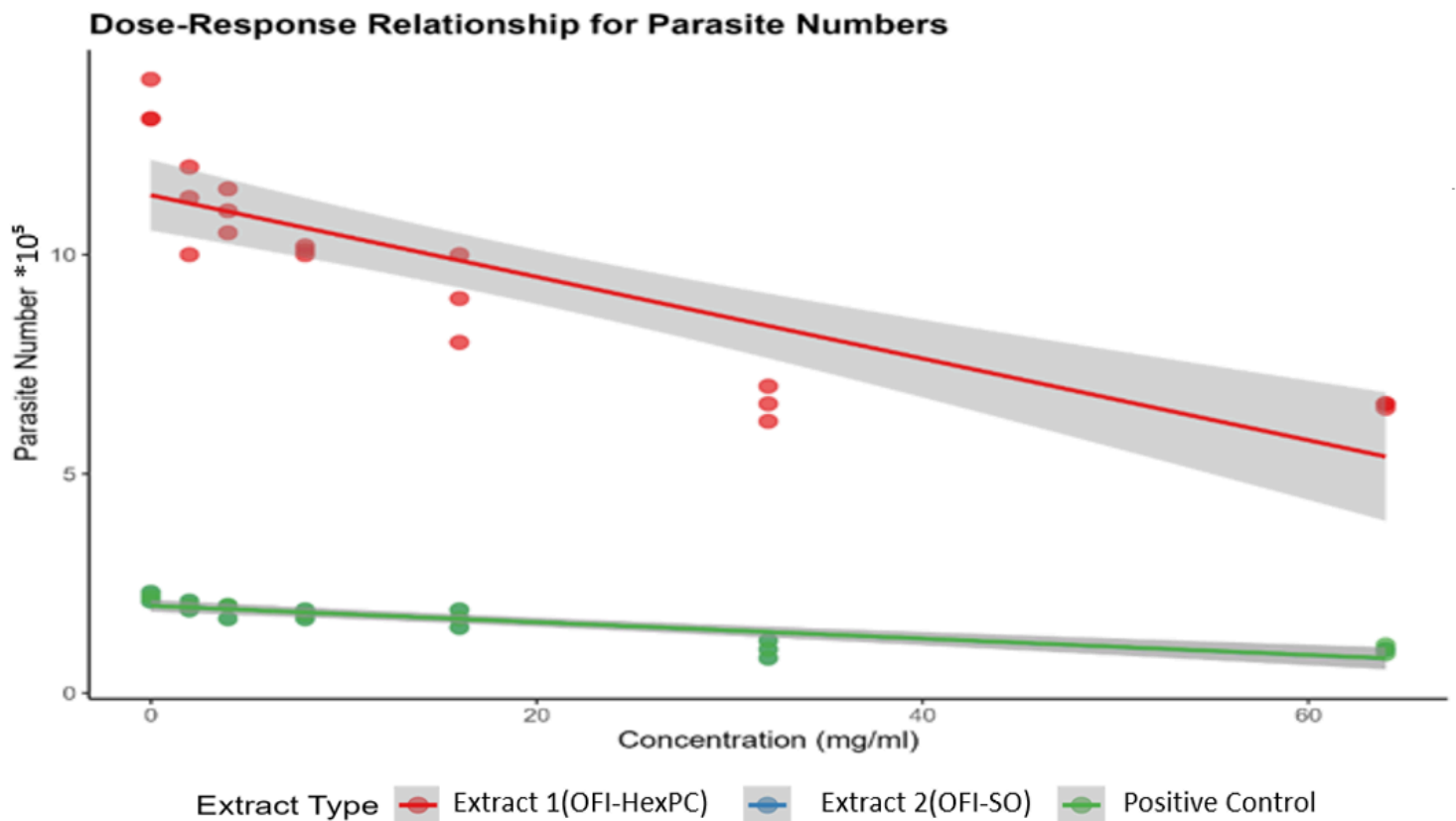
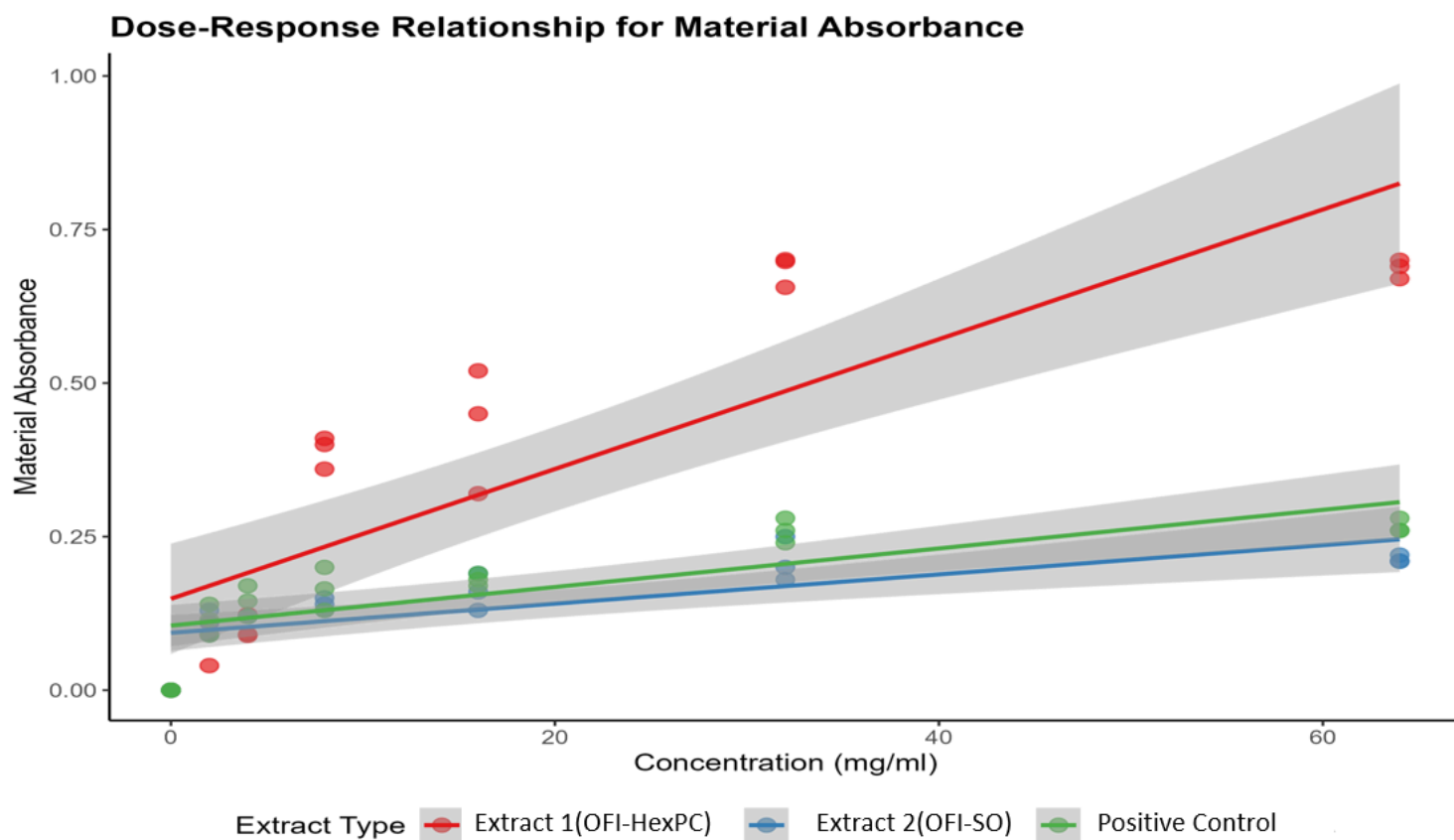


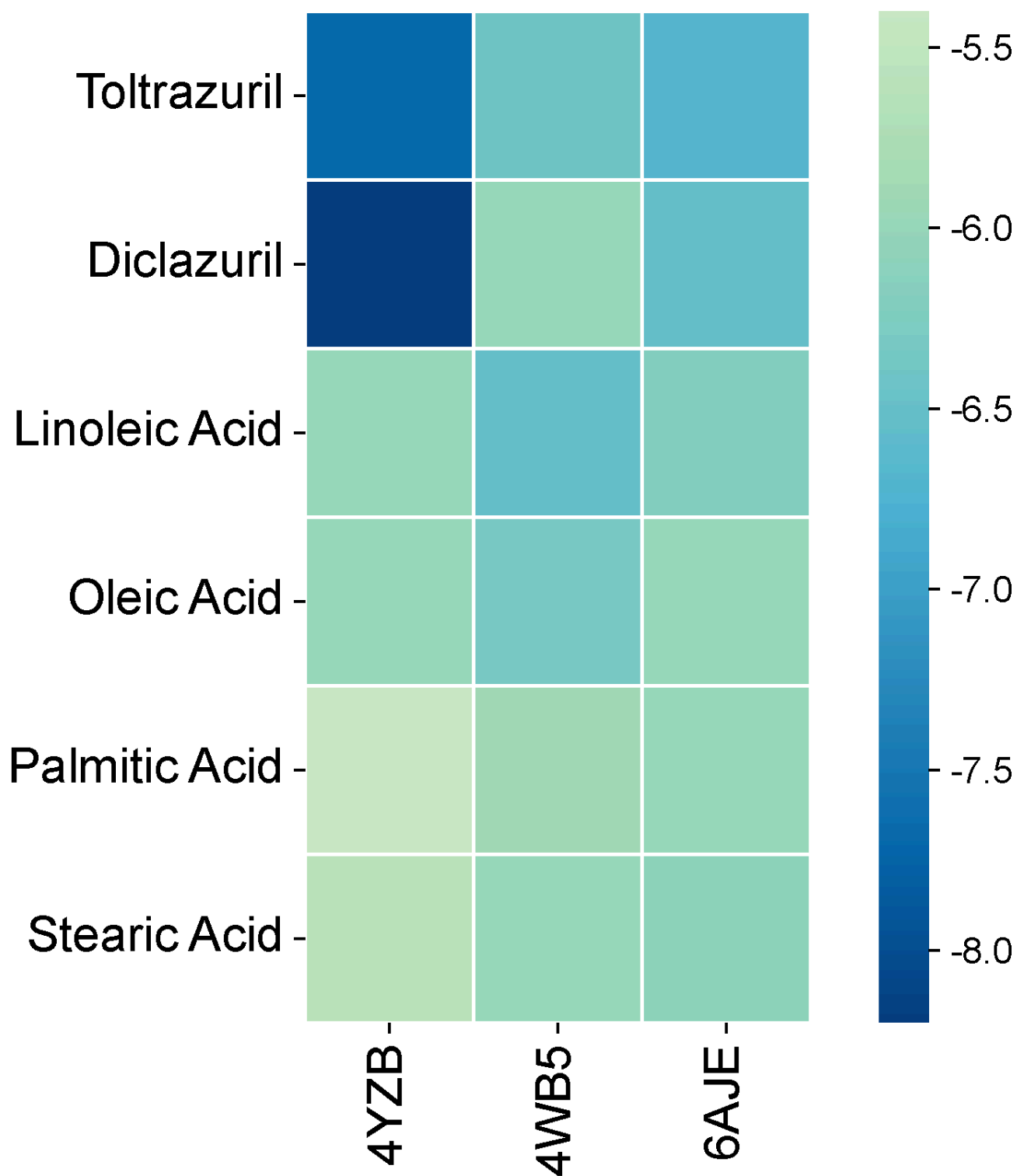
Figure 6

Scatter plot showing the variation in parasite numbers according to extract concentration, illustrating the dose-response relationship for each extract type



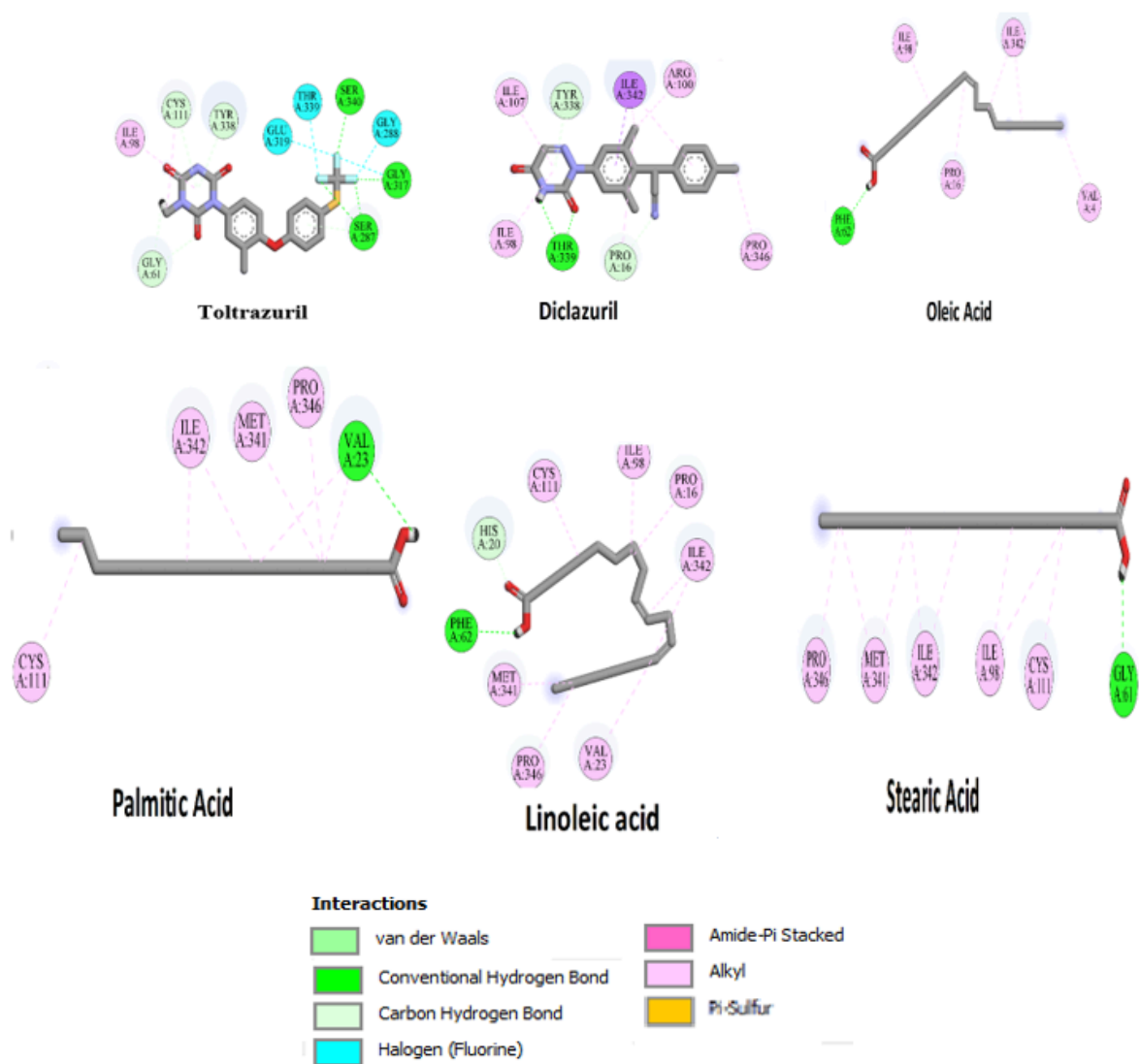
**Figure 7**

Scatter plot showing the variation in material absorbance according to extract concentration, illustrating the dose-response relationship for each extract type



**Figure 8**

Binding Scores and Major Compounds Interacting with: (a) 4YZB: CDPK1 from *Eimeria tenella*, (b) 4WB5: human cAMP-dependent protein kinase A, and (c) 6AJE: DHODH



**Figure 9**

The interactions and binding modes of Toltrazuril, Diclazuril, and major compounds with 6AJE

4YZB

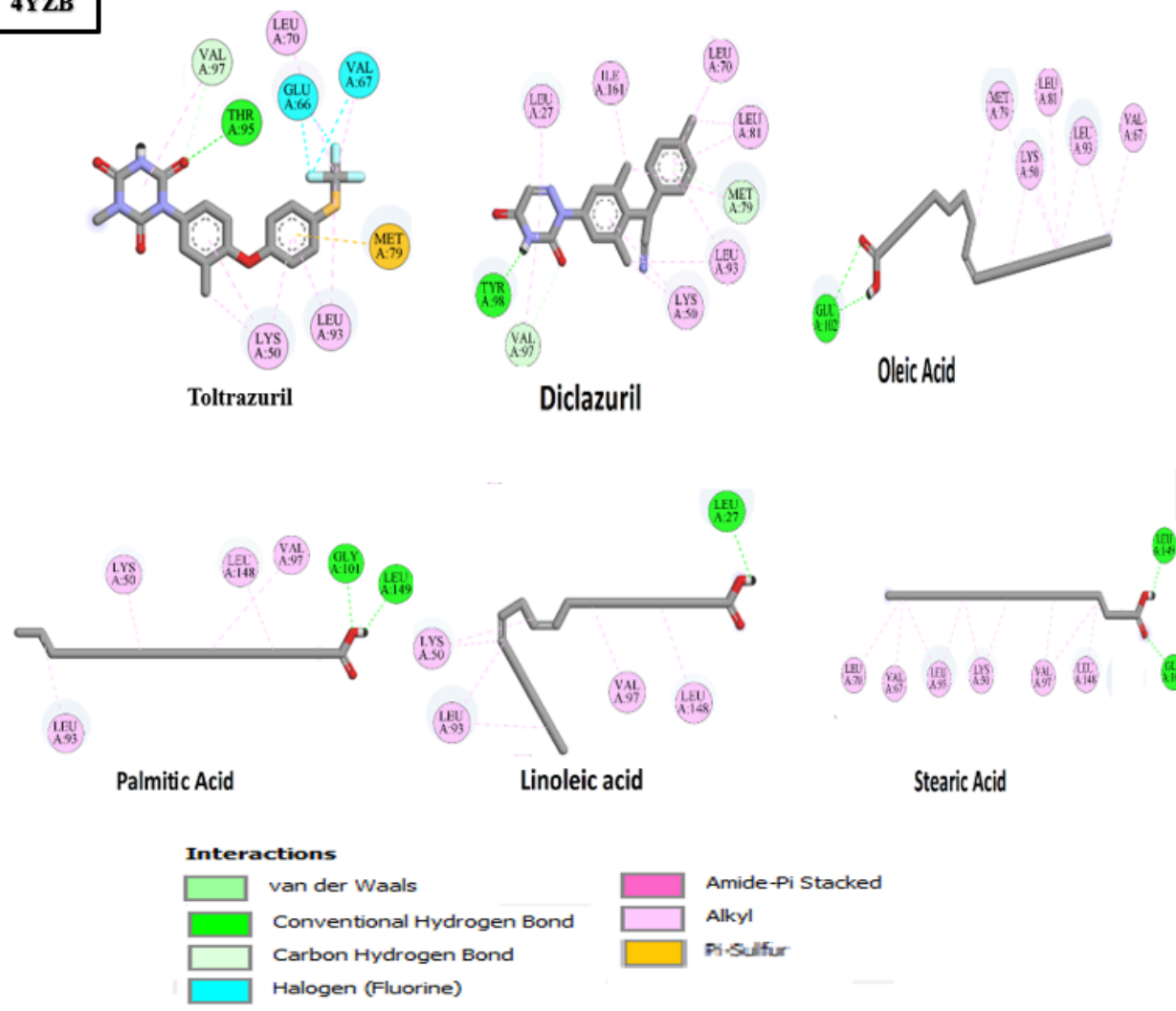
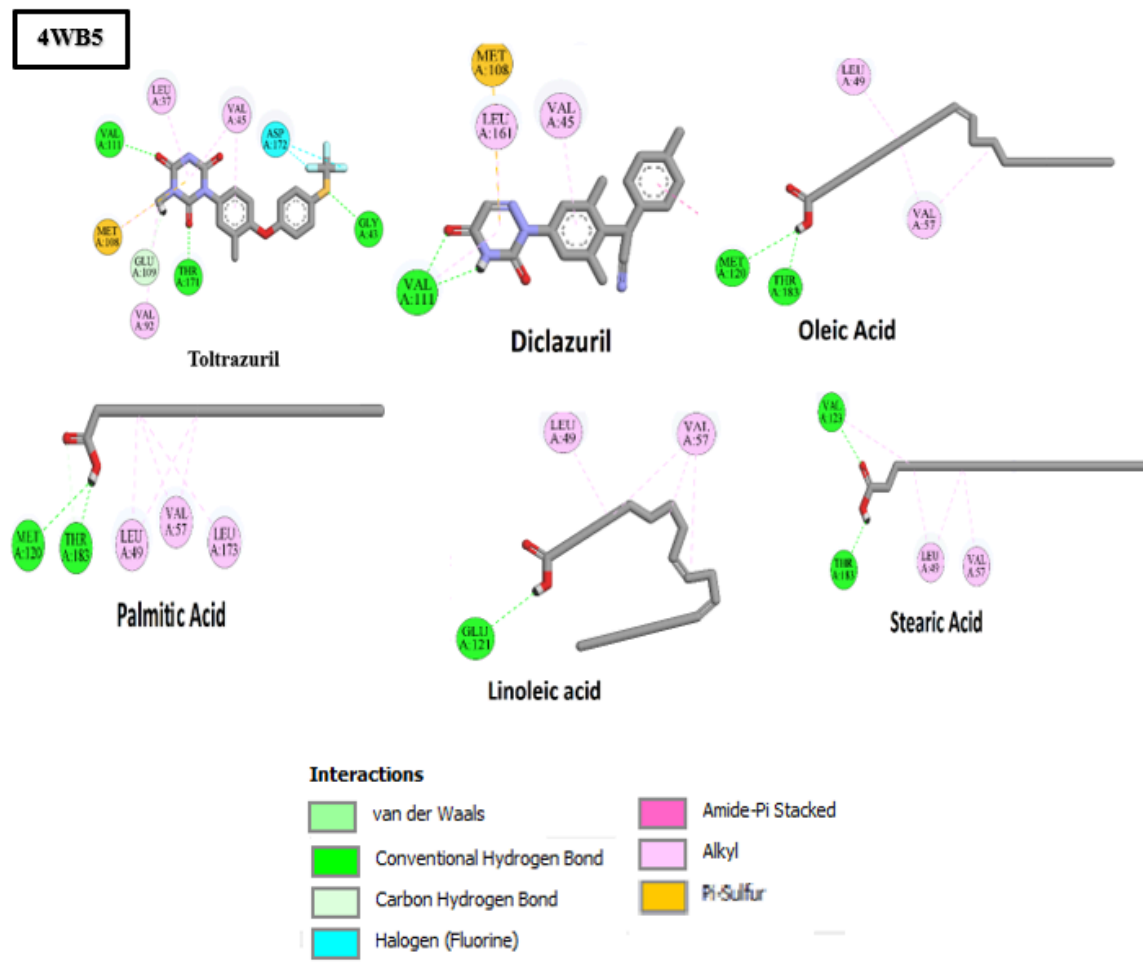


Figure 10

The interactions and binding modes of Toltrazuril, Diclazuril, and major compounds with 4YZB



**Figure 11**

The interactions and binding modes of Toltrazuril, Diclazuril, and major compounds with 4WB5