In vitro and *in silico* anticoccidial activity of the seeds of *Lepidium sativum* red and black varieties

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Research Article

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Abstract

Coccidiosis is a parasitic disease of major economic importance in the poultry industry. The exploration of natural alternative strategies such as phytochemicals is becoming significant in controlling coccidiosis. Therefore, in the present study, we investigate the in vitro anticoccidial activity of the seeds of red and black Lepidium sativum varieties that have multiple traditional medicinal uses and identify their major active components. The seeds of red and black L. sativum varieties were extracted with 80% methanol and tested for their in vitro anticoccidial activity using a sporulation inhibition assay. The 80% methanol extract of the red variety was solvent partitioned into four fractions, and each was tested for activity. Then, column chromatography of the active fractions was carried out to isolate compounds. The chemical structures of the compounds were determined by spectroscopic methods (^{1}H, ^{13}C and DEPT NMR). The 80 methanol extract of both the black and red varieties inhibited sporulation of oocysts by 83.71% and 98.27% at concentrations of 1000 mg/ml, respectively. Among all tested substances, the chloroform fraction, followed by the hexane, was found to be the most active one with sporulation inhibition values of 93.5% and 98.91% at 500 and 1000 mg/ml doses, respectively. Moreover, linoleic acid, alpha-linolenic acid and methyl sinapate were identified and characterized as the major components of the active fractions. Furthermore, molecular docking and conformational similarity scanning study of the compounds and anticoccidial drugs revealed that methyl sinapate had a strong binding affinity and conformational alignment to dihydrofolate reductase (HYDE score, -41.6 KJ/mol) and trimethoprim (FlexS scores, 0.84), respectively. In addition, to find further chemically similar analogs of methyl sinapate, we navigated the chemical space library of billions of commercially available synthetic compounds using the bioinformatics tool infiniSee 5.0.1 and identified 100 molecules with FlexS scores in the range of 0.67–0.78. These findings along with the safety profile derived from the acute oral toxicity tests of the extract suggest L. sativum as an alternative source of treatment for coccidiosis.

Introduction

Coccidiosis, a disease caused by protozoal parasites from the genus Eimeria, is of major economic importance in the poultry industry, seriously affecting the development of growing chickens (Williams, 2005; Conway and Mckenzie, 2007). It is the second largest cause of morbidity and mortality of chicks following bacterial diseases, whenever the poultry is reared in crowding conditions (Ballweber, 2001; Adamu et al., 2013). Globally, the annual economic losses due to coccidiosis are estimated to be 3 billion. Anticoccidial drug supplements are a good preventive measure and are well adapted to large-scale use. When preventive measures fail, anticoccidial treatment regimens are issued as a last resort. In general, two broad categories of anticoccidial drugs are utilized. These are polyether antibiotics or ionophores and synthetic antimicrobials that belong to five classes: DNA synthesis inhibitors —dihydroorotate dehydrogenase, dihydrofolate reductase, dihydropteroate synthase inhibitors, and thiamin transport inhibitors; and protein synthesis inhibitors (Greif et al., 2001). Most of these classes of anticoccidial drugs are also human medicine. For this reason, they are banned in commercial poultry production to avoid the potential development of antibiotic-resistant bacteria. As a result, non-antibiotic feed additives are developed and utilized nowadays to improve broiler performance (Brenes and Roura, 2010). Nevertheless, due to the prolonged use, coccidial resistance has been reported with almost all commercially available drugs, including ionophores (Chapman, 1997; Allen and Fetterer, 2002). Therefore, there is currently an urgent need for novel and alternative approaches to control and treat c*occidiosis* (Dalloul and Lillehoj, 2005). In this regard, the exploration of medicinal plants' secondary metabolites which have a long history of use, safety, and efficacy is an important alternative strategy. Here, we investigate the *in vitro* anticoccidial activity of the seeds of red and black *Lepidium sativum* varieties that have multiple local traditional medicinal applications for various human and animal ailments such as diarrhea, dysentery, unidentified gastrointestinal disorders, stomach-ache, indigestion, febrile disease, and skin disorders (Gedif and Hahn, 2003; Teklehaymanot et al., 2007). We also identified and characterized the major active components of the plants and studied their safety profiles and *in silico* mechanism of action.

Methods

Chemicals and instruments

Chromatographic separation was performed on column silica gel (0.063-0.200 mm, 70-230 mesh, Merck KGaA, Darmstadt, Germany) and on analytical thin layer silica gel (Silica gel 60 F254, 0.2 mm thick). Potassium dichromate (BDH Chemicals Ltd., England) and solvents (hexane, chloroform, methanol, and butanol) were used for extraction, fractionation, and activity testing.

NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a Bruker Avance DMX400 FT-NMR spectrometer (Bruker, Billerica, Massachusetts, USA) using tetramethylsilane (TMS) as internal standard. All spectra were measured in $CDCl_3$.

Plant material

The seeds of *L. sativum*, the red and black varieties, were purchased from Shola Gebeya, Addis Ababa, Ethiopia, in February 2022 and identified by Mr. Melaku Wondafrash, a senior botanist at the National Herbarium, Addis Ababa University (AAU), Addis Ababa, Ethiopia, where voucher specimens were deposited (Collection number: MA001).

Extraction, fractionation and isolation

1 Kg seeds of red and black *L. sativum* varieties were defatted separately with hexane. Then the seeds were macerated in 80% methanol at room temperature for 3 days with occasional shaking. Removal of the organic solvent using rotary evaporator (BUCHI Rotavapor™ R-300, *Switzerland)* followed by freeze-drying of the remaining water extract yielded 8 gm brown red and 7.7 gm dark solids for the red and black varieties consecutively. 5 gm of the dried extracts of both varieties were dissolved in 95% methanol and partitioned with hexane to collect the most nonpolar components (fraction I). The methanol phase was concentrated to dryness, dissolved in water and partitioned with chloroform. The chloroform layer was collected and concentrated in a rotary evaporator to give a dried solid designated crude phenolic fraction II. The aqueous layer was further portioned with butanol. The butanol was concentrated as fraction III and the remaining aqueous phase was freeze-dried as fraction IV. Fraction I was purified by gradient elution using hexane:ethyl acetate solvent system on silca gel column to give LSC-1 (120 mg). Furthermore, fraction II was applied on column silca gel chromatography and eluted with chloroform-methanol-NH_3 (90:10:1) solvent system to yield LSC-2 (58 mg).

In vitro anti-coccidial assay

Preparation and counting of oocysts

The fecal samples were collected from *Eimeria tenella* infected chicken ceca from the outbreaks at the Bishoftu/Debre Zeitpoultry farm. Oocysts were isolated using a flotation procedure described by Permin and Hansen, (1998), and counted, after isolation, the pellet was re-suspended in 5 ml of saturated salt solution. After vortexing for 1 min, a subsample was transferred into a McMaster chamber, using a disposable plastic pipette. The tube was vortexed again before the second chamber of the McMaster slide was filled with a second sub-sample. The preparation was left for 5 min before counting, allowing the oocysts to float up. All the oocysts under the grid of each chamber in the McMaster slide were counted using ×10 objectives and the mean of the two counts was calculated. This process was repeated twice and a count of one oocyst was equivalent to 300 (45 ml – the total volume of the original suspension of feces/0.15 ml – the volume of the sample examined) oocysts/sample (Haug et al. 2006; Taylor et al. 2007). The number of unsporulated oocysts in the original aqueous suspension of feces was adjusted to one hundred oocysts per ml.

In vitro sporulation inhibition assay

An *in vitro* assay was used to evaluate the effect of the extracts and fractions of *L. sativum* varieties on the sporulation of coccidian oocysts. Oocysts were sporulated by incubating a similar amount of concentrated suspension of oocysts (100

oocysts per ml) in distilled water and different substances with 2 ml of 2.5 percent of potassium dichromate solutions with forced aeration for 96 h (Conway and Mckenzie, 2007; Bowman, 2009). In this assay, the unsporulated oocysts were incubated with three concentrations of the test substances (250, 500, and 1000 mg/ml) and one with zero concentration of the test substances (or solvent vehicle only as negative control) for 96 h at 25–29 °C, using water baths to maintain a constant temperature. Three replications were made for each concentration including negative control. The oocysts were gently aerated with open air away from sunlight by stirring. At the end of the incubation, the incubated oocysts were washed twice in tap water and stored at 4 °C until being counted. The number of sporulated and non-sporulated oocysts was counted and the percent sporulation was estimated by counting the number of sporulated oocysts in a total of 100 oocysts. The whole experiment was repeated to confirm the results (Abbas et al., 2020).

Mean percent Sporulation and percent inhibition were calculated using the following formulae.

$$\% \text{ Sporulation } = \left(\frac{\text{Number of sporocysts}}{\text{Total number of count}}\right) \text{X } 100$$

$$\% \text{ Sporulation inhibition} = \left(\frac{\% \text{ Sporulation of negative control} - \% \text{ Sporulation of test substance}}{\% \text{ Sporulation of negative control}}\right) \text{X } 100$$

Acute oral toxicity testing

Acute oral toxicity study was conducted as per the internationally accepted protocol of OECD Guideline 425 (OECD, 2008). Ten healthy Swiss female albino mice weighing 20-25 g were randomly grouped into two each having 5 mice. Following 3-4 h of fasting (food only), one mouse from each group was orally administered 2000 mg/kg of the 80% methanol extract of red and black L. sativum varieties, consecutively. The mice were then observed individually for any physical or behavioral changes, such as loss of appetite, ruffled fur, lacrimation, mortality, and other signs of toxicity for 4 h. The same procedure was followed for the remaining mice for the next five consecutive days and the results were recorded. The follow-up observations were continued for all mice for 14 days.

Molecular docking study

Docking studies and conformational similarity scanning were carried out using SeeSar12.1.0 (Narcissus) software (BioSolvelT, Sankt Augustin, Germany). The crystal structures of four enzymes —mannitol-l-phosphate dehydrogenase (Protein Data Bank; PDB: 1LJ8), dihydropteroate synthase (PDB: 6DAY), dihydroorotate dehydrogenase (PDB: 6AJE), and dihydrofolate reductase (PDB: 4KM0)—selected for the study were loaded from the PDB in the protein mode. The ligands (including the inhibitors and coenzymes) found in the crystal structure of the proteins were identified, the binding sites were selected, and the binding modes were calculated. For 1LJ8, the coenzyme NAD binding pocket was selected for docking, and for 6DAY and 4KM0, the binding pockets of their inhibitors 4-aminobenzoic acid and pyrimethamine, respectively, were selected. Then, a list of all anticonicdal drugs, the inhibitors and coenzymes found in the crystal structure of the proteins, and isolated molecules were loaded as sdf files in the docking mode, and docking was carried out for the individual proteins. The best pose solutions were determined, the physiochemical parameters were calculated in the docking mode, and 3D conformational similarity scanning of molecules was carried out in the analyzer mode. Finally, infiniSee 5.0.1 was used to carry out similarity scanning or an analog search of our molecule of interest from the chemical space library, which contains more than 36 billion compounds. The HYDE score was used to estimate the binding affinity of the molecules. Likewise, FlexS sore was used to estimate their conformational similarity index (Schneider *et al.,* 2012; Schneider *et al.,* 2013; Beroza et al., 2022).

Statistical analysis

Data analysis was carried out using IBM SPSS (Statistical Package for Social Sciences) Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. Results were expressed as mean ± standard error of mean (M ± SEM). The statistical significance was determined by one-way ANOVA followed by Tukey post hoc test to compare percent sporulation among the treatment and control groups. $P < 0.05$ were considered significant.

Results

In vitro anti-coccidial activity of the hydroalcoholic extracts

As shown in figure 1, the hydroalcoholic extracts of both red and black *L. sativum* variants showed a significant sporulation inhibition effect at all concentrations tested (Fig. 1 and 2). The oocysts treated with 500 and 1000 mg/ml concentrations of the red *L. sativum* variant were able to sporulate only by 6.7333 and 1.5667 %, respectively in contrast to the negative controls which were able to sporulate by 91.0 %. As a result, the extracts of the red variety exhibited sporulation inhibition activity of 92.6 and 98.27% at the concentrations of 500 and 1000 mg/ml, respectively. Similarly, the black *L. sativum* variety showed only 18.27 and 14.66% oocyst sporulation compared to 90.03% sporulation in the untreated groups. Hence, it showed a 79.70 and 83.71% sporulation inhibition effect at 500 and 1000 mg/ml, respectively.

In vitro anti-coccidial activity of solvent fractions

Since the 80% extract of red *L. sativum* gave better activity, we further solvent fractionated it to pinpoint the active constituents. Table 1 shows the *in vitro* oocyst sporulation inhibition activity of the four fractions. Among them, the chloroform fraction was found to be the most active one, with sporulation inhibition values of 98.91% and 93.59% at 500 and 1000 mg/ml doses, respectively. In other words, only 5.7988% and 0.9813% of oocysts were able to sporulate in 500 and 1000 mg/ml chloroform treated groups, respectively. The hexane fraction inhibited the sporulation at all doses treated, with the highest being at 500 and 1000 mg/ml with 86.2810 and 89.23% inhibition. The aqueous fraction also exhibited mild inhibitory activity with 68.1107% inhibition at 1000 mg/ml. On the other hand, the butanol fraction exhibited minimal or no activity at all doses, showing greater than 79.76% oocyst sporulation.

Table 1. *In vitro* anti-coccidial activity of the solvent fractions of *Lepidium sativum* red variety against *Eimeria tenella* oocysts sporulation.

Test substances	Concentration (mg/ml)	Percent sporulation	Percent sporulation inhibition
Hexane fraction	250 mg/ml	31.3053 ± 0.5847 b*c*m*	65.4276
	500 mg/ml	12.4225±0.5798 a*c**m*	86.2810
	1000 mg/ml	9.7494± 0.6936 a*b**m*	89.2331
	250 mg/ml	11.2278± 0.4815 e*f*m*	87.6004
Chloroform fraction	500 mg/ml	5.7988± 0.7360 d*f*m*	93.5960
	1000 mg/ml	0.9813± 0.5449 d*e*m*	98.9162
	250 mg/ml	84.4549± 0.3345 h**i*m*	6.7311
Butanol fraction	500 mg/ml	80.2063±0.9264 g**i***m*	11.4231
	1000 mg/ml	79.7644± 0.4960 g*f***m*	11.9112
	250 mg/ml	68.1347± 0.5764 k*l*m*	24.7546
Aqueous fraction	500 mg/ml	61.8151± 0.7162 j*l*m*	31.7337
	10000 mg/ml	35.2142± 0.7151 j*l*m*	68.1107
Negative control	0 mg/ml	90.5500 ± 0.5836 a-l*	0.0000

Values are presented as mean ± SEM; n = 3; a = compared to hexane fraction 250 mg/ml, b = compared to hexane fraction 500 mg/ml, c = compared to hexane fraction 1000 mg/ml l, d = compared to chloroform fraction 250 mg/ml, e = compared to chloroform fraction 500 mg/ml, f = compared to chloroform fraction 1000 mg/ml, g = compared to butanol fraction 250 mg/ml, h = compared to butanol fraction 500 mg/ml, i = compared to butanol fraction 1000 mg/ml, j = compared to aqueous fraction 250 mg/ml, k = compared to aqueous fraction 500 mg/ml, l = compared to aqueous fraction 1000 mg/ml, m = compared to negative control; * ($p < 0.001$), ** ($p<0.01$)

Acute oral toxicity

Acute oral toxicity test results of this study documented that the 80% methanol extracts of *L. sativum* red and black varieties were safe at a dose of 2000 mg/kg [21, 37]. After 72 hours, the animals tolerated the administered dose although immediate mild toxicity signs such as ruffled fur, loss of appetite and slight sleepiness, which disappeared a few hours after administration were observed. Also, there was no mortality within 14 days of observation which entails that the LD_{50}s of the extracts are above 2000 mg/kg.

Structural elucidation of the isolated compounds

In an attempt to characterize the major constituents of the active fractions, hexane and chloroform, we isolated three compounds (**1-3**) using column chromatography. Compound **1** was obtained as a light green crystal. The ^{1}H NMR spectrum of compound **1** (Fig. S1) shows five distinct proton signals, three singlets and two doublets. The singlet peaks at δ_H 3.829 (6H, *s*) and 3.739 (3H, *s*) are deshielded oxygenated methyl protons. The integral of the CH_3 peaks at δ_H 3.829 was twice the other peak at δ_H 3.739, indicating that it was composed of six chemically equivalent symmetric CH_3 protons. The two doublet signals at δ_H 7.534 (1H, *d*, *J* = 18 Hz, H-7) and 6.245 (1H, *d*, *J* = 18 Hz, H-8) are alkene CH protons. The larger coupling constants of the two doublets signify that they are trans coupled. The remaining singlet peak at 6.691 (2H, *s*, H-2, H-6) is anaromtic CH protons. The integral of this aromatic proton peak which showed two hydrogens indicated that it was composed of two chemically equivalent symmetric CH aromatic protons. The ^{13}C and DEPT spectra (Fig. S1) of the compound support the proton data. The ^{13}C shows a total of nine carbon signals. The deshielded carbon peaks at δ_C 56.22 and 51.60 are due to the oxygenated methyl carbons. Likewise, the two carbon signals at δ_C 145.22

and 115.25 are the trans alkene CH carbons. The remaining four aromatic carbons and the carbonyl carbon signals are found at δ_C 105.04, 125.66, 137.23, 147.23, and 167.67, respectively. Moreover, the aromatic carbon peaks at δ_C 147.23 and 105.04 show pairs of two chemically equivalent symmetric CH aromatic carbons. In DEPT spectra, the absence of three aromatic carbons at δ_C 125.66, 137.23, and 147.23 and the carbonyl carbon δ_C at 167.67 signals confirm that they are quaternary carbons. Therefore, based on these data and in comparison with the reported ^{1}H and ^{13}C NMR results of the same compound (Choudhary et al., 1988; Dall'Acqua et al., 2002), the structure of compound **1** was determined to be methyl sinapate or (E)-methyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate. Table 2 summarizes the NMR data of compound **1**.

Table 2. ^{1}H and ^{13}C NMR data of compound **1** measured in methanol-d_4

Present data			Reference data (Fugita et al., 1984)	
Position	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
1	125.66	-	124.7	-
2	105.04	6.691 (1H, *s*)	106.3	7.01 (1H, *s*)
3	147.23	-	148.2	-
4	137.23	-	138.7	-
5	147.23	-	148.2	-
6	105.04	6.691 (1H, *s*)	106.3	7.01 (1H, *s*)
7	145.22	7.534 (1H, *d*, J = 18 Hz)	145.5	7.58 (1H, *d*, J = 16 Hz)
8	115.25	6.245 (1H, *d*, J = 18 Hz)	114.8	6.60 (1H, *d*, J = 16 Hz)
$\underline{CH_3}OArO\underline{CH_3}$	56.22	3.829 (6H, *s*)	56.2	3.82 (6H, *s*)
$CO\underline{CH_3}$	51.60	3.739 (3H, *s*)	51.3	3.71 (3H, *s*)
$\underline{C}OCH_3$	167.67	-	167.3	-

s = singlet, *d* = doublet

Compounds **2** and **3** were isolated as a yellow, oily liquid. In the ^{1}H NMR spectrum of the compounds **2** and **3** (Fig. S2), the first two triplet signals in the up field region at δ_H 0.880 (3H, *t*, H-18) and 0.996 (3H, *t*, H-18) correspond to the CH_3 methyl protons of **2** and **3**, respectively. The next multiplet peak in the up field at δ_H 1.285 (8H, 8H, *m*, H-4—H-7) was the CH_2 protons of C-4 to C-7 of the long chain alkyl groups of the compounds. Similarly, the proton signals from δ_H 1.620 (2H, *m*, H-3) to δ_H 2.812 (4H, *m*, H-8 and H-17) belong to the remaining CH_2 protons of the compounds. Furthermore, the CH protons peaks of the compounds were found at δ_H 5.334 (2H, *m*, H-9 and H-13) and 5.376 (2H, *m*, H-10 and H-12) for compound **2**, and at δ_H 5.334 (2H, *m*, H-9 and H-16), 5.354 (2H, *m*, H-12 and H-13), and 5.376 (4H, *m*, H-10 and H-15) for compound **3**. The details of the proton data are given in table 3. The ^{13}C and DEPT spectra of compounds (Fig. S2) back the proton data. In the ^{13}C spectra, the methyl carbons are shown at δ_C 14.01 and 14.20 for compounds **2** and **3**, respectively. The ^{13}C peaks from δ_C 20.51 to δ_C 34.05, which are also shown as negative signals on the DEPT spectrum, correspond to the CH_2 protons of the compounds. Likewise, the CH protons for the compounds are found from δ_C 17.07 to δ_C 131.70 as positive signals on DEPT spectrum. The carbonyl carbons of both compounds resonate at δ_C 180.38 in ^{13}C spectrum and, as expected, is absent on the DEPT spectrum. Thus, based on these data and in comparison with previous results (Takaya et al., 2003; Hatzakis et al., 2011), the structure of the compounds **2** and **3** are identified to be linoleic acid and alpha-linolenic acid or (9E,12E)-octadeca-9,12-dienoic acid and (9E,12E,15E)-octadeca-9,12,15-trienoic acid, respectively. Table 3 summarizes the NMR data of compounds **2** and **3**.

Table 3. ^{1}H and ^{13}C NMR data of compound **1** measured in methanol-d_4

Compound **2**					Compound **3**			
Present data			Reference data (Hatzakis et al., 2011)		Present data		Reference data (Takaya et al., 2003)	
Position	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
1 $\underline{C}OCH_3$	180	-	173.83	-	180	-	180	-
2	34.06	2.318 (2H, *t, J* = 7..3Hz)	34.23	2.32 (2H, *t, J* = 7.53 Hz)	34.06	2.318 (2H, *t, J* = 7..3Hz)	34.0	2.27 (2H, *t, J* = 7.4Hz)
3	24.82	1.636 (2H, *m*)	24.84	1.62 (2H, *m*)	24.63	1.620 (2H, *m*)	24.8	1.57 (2H, *m*)
4	29.09	1.285 (2H, *m*)	29.06	1.30 (2H, *m*)	29.09	1.285 (2H, *m*)	29.05	1.28 (2H, *m*)
5	29.19	1.285 (2H, *m*)	29.16	1.30 (2H, *m*)	29.19	1.285 (2H, *m*)	29.11	1.28 (2H, *m*)
6	29.47	1.285 (2H, *m*)	29.29	1.30 (2H, *m*)	29.43	1.285 (2H, *m*)	29.29	1.28 (2H, *m*)
7	29.69	1.285 (2H, *m*)	29.66	1.30 (2H, *m*)	29.67	1.285 (2H, *m*)	29.57	1.28 (2H, *m*)
8	27.17	2.069 (2H, *m*)	27.14	2.04 (2H, *m*)	27.20	2.050 (2H, *m*)	27.2	2.05 (2H, *m*)
9	129.96	5.328 (1H, *m*)	129.98	5.28–5.40 (1H, *m*)	129.96	5.328 (1H, *m*)	130.2	5.33 (1H, *m*)
10	128.01	5.382 (1H, *m*)	128.04	5.28–5.40 (1H, *m*)	127.72	5.382 (1H, *m*)	127.8	5.33 (1H, *m*)
11	25.57	2.783 (2H, *m*)	25.60	2.76	25.57	2.797 (2H, *m*)	25.6	2.78 (2H, *m*)
12	127.85	5.382 (1H, *m*)	127.86	5.28–5.40 (1H, *m*)	128.14	5.354 (1H, *m*)	128.2	5.33 (1H, *m*)
13	130.00	5.328 (1H, *m*)	130.19	5.28–5.40 (1H, *m*)	128.11	5.354 (1H, *m*)	128.0	5.33 (1H, *m*)
14	27.14	2.087 (2H, *m*)	27.12	2.04 (2H, *m*)	25.47	2.812 (2H, *m*)	25.5	2.78 (2H, *m*)
15	29.31	2.019 (2H, *m*)	29.29	2.04 (2H, *m*)	127.07	5.37 (1H, *m*)	127.01	5.33 (1H, *m*)
16	31.55	2.005 (2H,*m*)	29.29	2.04 (2H, *m*)	131.70	5.34 (1H, *m*)	131.9	5.33 (1H, *m*)
17	22.72	1.603 (2H, *m*)	22.54	1.62 (2H, *m*)	20.51	2.035 (2H, *m*)	20.50	2.05 (2H, *m*)
18	14.01	0.880 (3H, *t*)	14.04	0.88 (3H, *t*)	14.20	0.996 (3H, *t*)	14.20	0.95 (3H, *t*)

s = singlet, *d* = doublet, *m* = multiplet, , *t* = triplet

Molecular docking study

To get further insight into the mechanism of action of the constituents of the active extracts and fractions of *L. sativum*, we carried out molecular socking and conformational similarity scanning of the isolated compounds (**1-3**), 18 anti-coccidial drugs belonging to three classes and PABA on four plausible targets (Table S1). Mannitol-l-phosphate dehydrogenase (1LJ8) was chosen as it is critical enzyme involved in the mannitol cycle crucial to the sporulation of the oocysts (Allocco et al., 1999). Dihydropteroate synthase (6DAY), dihydroorotate dehydrogenase (6AJE), and dihydrofolate reductase (4KM0) were selected because they are the plausible targets of the anti-coccidial drugs (Greif et al., 2001). The docking result showed that among the four targets methyl sinapate has a strong binding affinity for dihydrofolate reductase, with the highest top score of the screened molecules and an estimated HYDE score of -41.6 KJ/mol. The similarity scanning result was also in agreement with the docking showing methyl sinapate has similar conformational alignment to trimethoprim, a dihydrofolate reductase inhibitor; they are nearly perfectly superposed on each other with a FlexS score of 0.84. The binding mode of methyl sinapate (compound **1**) is shown in Figure 3. It forms two hydrogen

bonds with Gln28 and Ile94 amino acid residues in the binding site of 4KM0. The para-hydroxyl hydrogen at C-4 of the benzene ring of compound **1** and its carbonyl oxygen form hydrogen bonds with the backbone carbonyl oxygen of Ile94 and the side chain amide hydrogen of Gln28, respectively. On the other hand, it had minimal binding affinity to dihydropteroate synthase and no binding affinity to dihydroorotate dehydrogenase or mannitol-l-phosphate dehydrogenase. Furthermore, linoleic acid (**2**) and alpha-linolenic acid **(3**) showed binding affinity to dihydroorotate dehydrogenase with scores of -34 KJ/mol and-32 KJ/mol, respectively. The carboxylic group of both compounds forms a hydrogen bond with the side chain guanidinium group of Arg103. However, they displayed slight affinity to dihydrofolate reductase, mannitol-l-phosphate dehydrogenase, and dihydropteroate synthase. In addition, they displayed no binding conformational similarity with other molecules. Finally, to find further novel chemically similar analogs of methyl sinapate, we searched the chemical space library of billions of commercially available synthetic compounds using the bioinformatics tool infiniSee 5.0.1 and identified 100 molecules with FlexS scores in the range of 0.63 to 0.79 (Table S2).

Discussion

In the present study, the 80% methanol extracts of *L. sativum* varieties as well as their fractions, specifically the hexane and chloroform fraction of the red variety, displayed *in vitro* sporulation inhibition anti-coccidial activity. We chose 80% methanol as an extractant solvent since it is capable of extracting polar, medium-polar and non-polar constituents of the seeds. Among the four fractions tested, the relatively non-polar polar fraction chloroform followed by the hexane showed superior activity indicating the major active constituents to be medium polar to non-polar compounds. Consequently, linoleic acid, alpha-linolenic acid and methyl sinapate were identified as the major components. Previously, the seed, leaf, stem, and root extracts of *L. sativum* were reported to have antimicrobial (El-Haggar et al., 2021), anticancer (Mehmood et al., 2011)**,** antioxidant, anti-inflammatory (Alqahtani et al., 2019), antidiabetic (Eddouks et al., 2005), hepatoprotective (Rajab and Ali, 2020), gastroprotective (Mehmood et al., 2011), antidiarrheal, antispasmodic (Rehman et al., 2012), and bone fracture healing activities. Moreover, a few imidazole alkaloids (Maier et al., 1998), phenolic compounds such as flavonoids (Fan et al., 2014; Kaiyrkulova et al., 2019) and polyunsaturated fatty acids have been isolated from the plant. Except for the antioxidant activity, the link between the isolated natural products and the biological activities of the extracts of the plant has not been established since the phytochemical and biological activity investigations of the extracts were carried out separately. However, here we followed a bioassay guided approach to isolate and characterize major compounds from the most active chloroform fraction, leaving the inactive polar fractions. Interestingly, one of the compounds methyl sinapate (1) is reported from the plant for the first time. Besides, supplementation with fats containing high concentrations of ω-3 fatty acids (α-linolenic acid (compound 2), eicosapentaenoic acid, and docosahexaenoic acid) such as fish oils effectively reduced lesions and the degree of parasitization and development causing ultrastructural degradation in both asexual and sexual stages (Danforth et al., 1997). The mechanism of these ω-3 fatty acids has been attributed to their oxidant nature due to the presence of a higher number of easily oxidized double bonds that induce a state of oxidative stress to the parasite damaging its development. Similarly, feed supplementation with antioxidants such as tocopherol and curcumin alone or in combination with anti-coccidial drugs reduced the intestinal infections of different *Eimeria* species (Allen et al., 1998). Thus, methyl sinapate, which has a documented antioxidant and anticancer effect, could play a major role in the sporulation suppression of the chloroform fraction of *L. sativum* (Nićiforović N and Abramovič, 2014). The *in silico* study also gave further molecular insight into its possible mechanism of action, strongly suggesting it to be a dihydrofolate reductase inhibitor, and this will aid future design and development of better semisynthetic derivatives of the compound for *coccidiosis*.

Conclusion

In conclusion, the seed extracts and solvent fractions of *L. sativum* exhibit *in vitro* anti-coccidial activity against *E. tenella* oocysts sporulation. Methyl sinapate, alpha-linolenic acid and linoleic acid are characterized to be the major components of the active fractions. The molecular docking study also identified possible targets, binding motifs and hence the

mechanism of action of methyl sinapate, showing its interaction with important amino acid residues in the binding site of the enzyme. These findings along with the safety profile derived from the acute oral toxicity tests suggest *L. sativum* as an alternative source of treatment for *coccidiosis*.

Declarations

Author contribution

Yonatan Alebachew: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Writing - original draft, review & editing. **Kebede Wondu**: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Writing - original draft, review & editing. **Tigist Getachew**: Data curation; Investigation. **Debora Tilahun**: Data curation; Investigation. **Meskerem Adamu**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing - review & editing. **Tsige Gebre-Mariam**: Conceptualization; Methodology; Project administration; Resources; Supervision; Validation; Writing - review & editing.

Competing interests

The authors declare no conflict of interest.

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Supplementary data

The ^{1}H, ^{13}C and DEPT NMR spectra of the compounds are available in the supplementary figures (Figs. S1 and S2). The physicochemical prediction of the isolated compounds, anti-coccidial drugs, and chemical space analogs of methyl sinapate are provided in supplementary tables (Tables S1 and S2). Table S2 is provided as sdf file.

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Figures

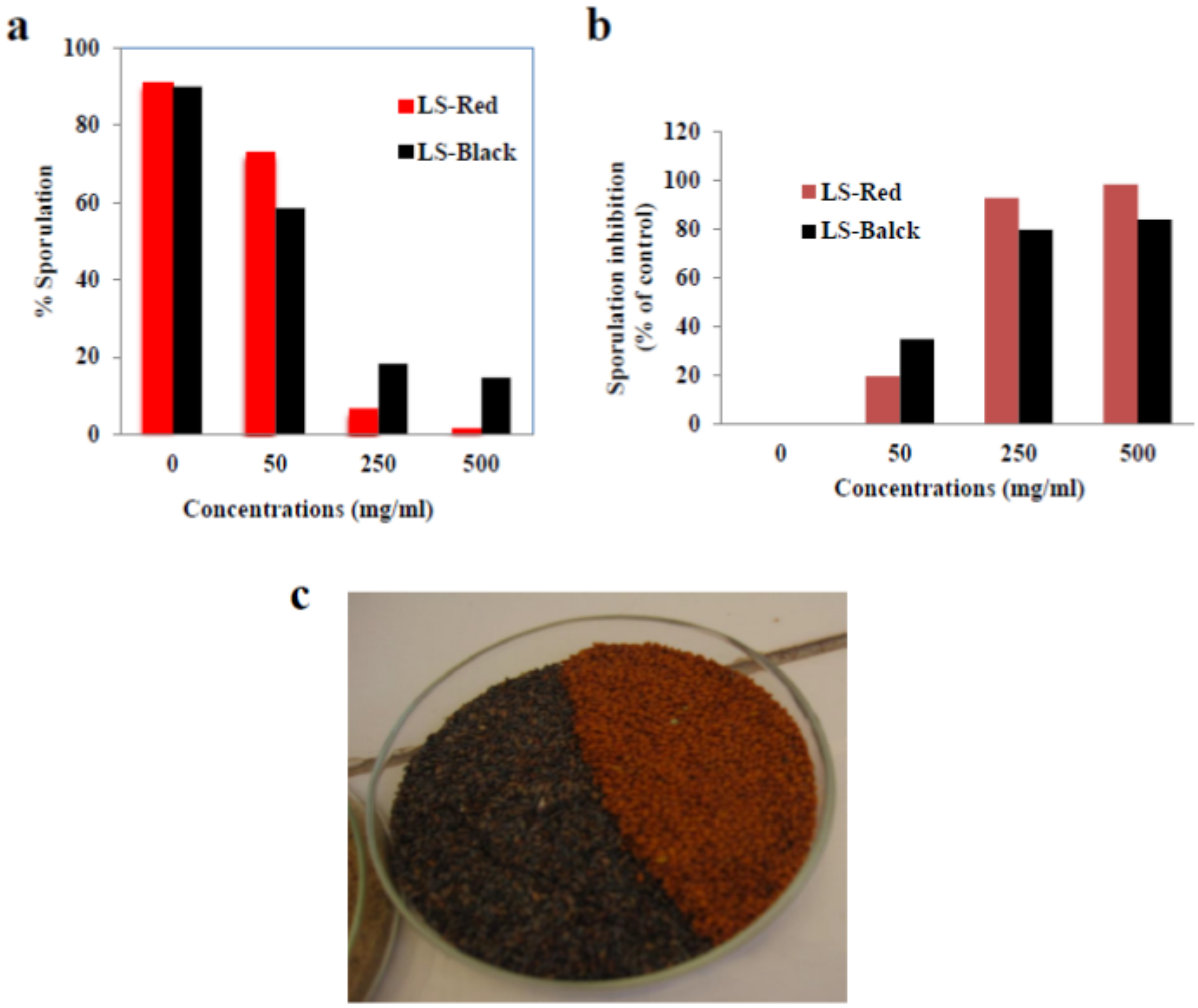

Figure 1

In vitro anti-coccidial activity of the 80% methanol seed extracts of *Lepidium sativum* red and black variety against *Eimeria tenella* oocysts sporulation. a) % Sporulation b) Sporulation inhibition c) Seeds of *Lepidium sativum,* red and black varieties

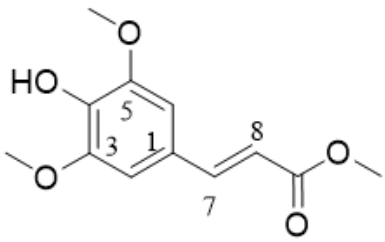

Figure 2

Fig. 3 Chemical structures of methyl sinapate (**1**)

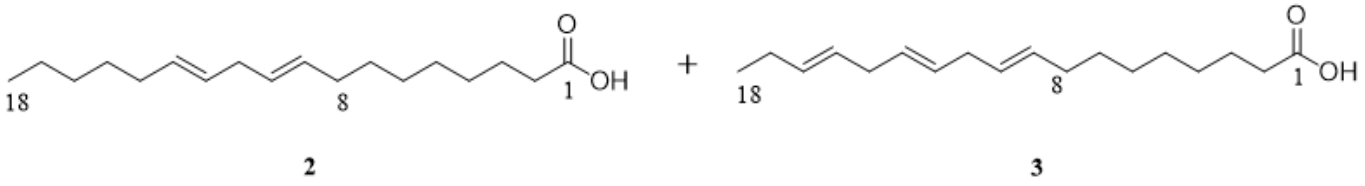

Figure 3

Chemical structures of linoleic acid (**2**) and alpha-linolenic acid (**3**)

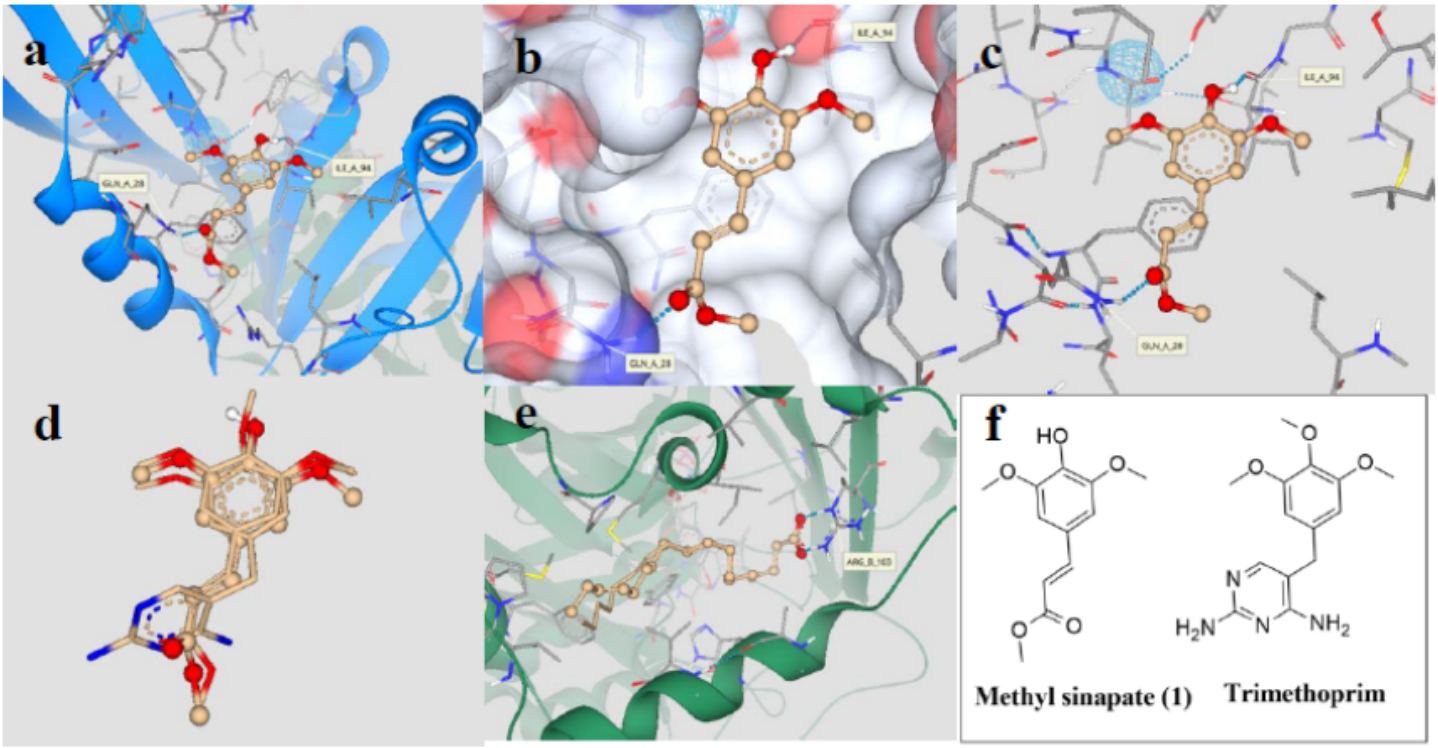

Figure 4

Figure 2: The binding modes of the compound**s** with dihydrofolate reductase (PDB 4KM0) and dihydroorotate dehydrogenase (PDB: 6AJE). **a**) Ribbon diagram showing binding interaction of compound **1** with amino acid residues of 4KM0. **b**) Surface representation and **c**) binding interaction of compound **1** in the binding site of 4KM0 with lipophilicity coloring, white representing hydrophobic pockets and blue representing hydrophilic pockets. **d**) Superimposition of compound **1** (shown in ball-stick model) with trimethoprim (shown as a solid line) in the binding site of 4KM0. **e**) Ribbon diagram showing binding interaction of compound **2** and **3** with amino acid residues of 6AJE. **f**) Chemical structures compound **1**(methyl sinapate) and trimethoprim.

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