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Analysis of Secondary Metabolites (SMs) in *Ochradenus baccatus* Collected from Riyadh, Saudi Arabia and its Potential Preventive Role on Coccidia that Infect Rabbits

Hossam M. Aljawdah[1], Mutee Murshed[1], Aiman A. Ammari[1], Saleh N Maodaa[1], Saleh Al-Quraishy[1]

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ABSTRACT

Background: The increasing focus on medicinal and aromatic plants is due to their secondary metabolites (SMs), which offer a feasible alternative to synthetic pharmaceuticals. *O. baccatus* has been historically employed in folk medicine for its anti-inflammatory and antibacterial properties. Rabbits serve as a protein source and contribute to ecological balance.

Methods: A study was performed on the phytochemical composition of the extract from *O. baccatus*. The antioxidant activity of *O. baccatus* extract was assessed *in vitro* using ABTS assays and the IC_{50} values were determined. The antiparasitic activity *in vitro* was evaluated using five distinct concentrations of *O. baccatus* extract. The extract's inhibition of sporulated oocysts was evaluated after 72 hours.

Result: The FT-IR analysis of the *C. spinosa* extract revealed the presence of 12 distinct compounds. The GC-MS analysis identified approximately 11 primary biologically active compounds. The extract exhibited significant antioxidant properties, with inhibition rates varying from 93.655% to 17.255% across concentrations of 500 to 15.625 µg/mL. The IC50 value was determined to be 147.032 µg/mL. The antiparasitic effects were evaluated *in vitro*, revealing that oocysts exhibited the highest level of inhibition at concentrations of 50 mg/mL and 25 mg/mL.

Key words: Antioxidant, *Eimeria intestinalis*, *Ochradenus baccatus*, Phytochemical, SMs.

INTRODUCTION

The rising interest in medicinal and aromatic plants is attributed to their secondary metabolites (SMs), which present a viable alternative to synthetic pharmaceuticals. Their cost-effectiveness and reduced risks have facilitated their adoption in traditional medicine (Afroz *et al*., 2021; Ghorbanpour and Varma, 2017). The production and buildup of these chemicals are affected by genetic and environmental factors (Budiastuti *et al*., 2022; Hazrati *et al*., 2024).

The use of medicinal plants to treat a variety of illnesses (Bodeker and Ong, 2005). Herbs cure common health disorders like aches, pains, wounds, respiratory problems and musculoskeletal diseases (Kandpal *et al*., 2023).

Rabbits are utilized as a source of protein, as well as for ecological balance (Chen *et al*., 2024). These organisms act as reservoirs for numerous pathogens, with coccidiosis ranking among the most common diseases affecting rabbits (Murshed *et al*., 2024b). Coccidia of the genus *Eimeria* are common parasites in rabbits and one of the main causes of intestinal disorders on conventional rabbit farms (Murshed *et al*., 2024a). 15 species of *Eimeria* in rabbits have been identified. *E. perforans, E. piriformis, E. exigua, E. media, E. magna, E. coecicola, E. vejdovskyi, E. flavescens, E. roobroucki, E. intestinalis, E. agnosta, E. nagpurensis, E. irresidua, E. matsubayashi* and *E. oryctolagi,* parasitize the small intestine. These can be differentiated by the morphology of oocysts, site of infection, clinical signs and histopathological changes (Murshed *et al*., 2023).

[1]Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia.

Corresponding Author: Hossam M. Aljawdah, Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia. Email: haljawdah@KSU.EDU.SA
ORCIDs: 0009-0002-7484-0398, 0000-0003-3717-6424, 0000-0002-1900-8446, 0000-0002-0662-2113, 0000-0003-4204-3124

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Ochradenus baccatus Del., belonging to the Resedaceae family, is widely cultivated in Saudi Arabia, Ethiopia, Tunisia, Egypt, Morocco, Libya, Pakistan and other Middle Eastern countries. This plant is the most widespread species in the genus *Ochradenus* (Yousif *et al*., 2012). *O. baccatus* has traditionally been used as an anti-inflammatory and antibacterial agent in folk medicine (Al-Omar *et al*., 2020). The ethanolic extract of the plant has also been shown to have anti-inflammatory and anti-free radical activities. Also shown to fight cancer, parasites, helminths (Alqasoumi *et al*., 2012).

Environmental situations have a great effect on the biosynthesis and variability of secondary metabolites in plants (Camara *et al*., 2021). To our knowledge, scant data

has been published regarding the phytochemical analysis, antioxidant and antiparasitic properties of the aqueous methanol extract of the *O. baccatus* plant, which is employed in traditional medicine and harvested in Riyadh, Saudi Arabia. This study aims to analyze the disparities between the extracts investigated in different places and those of the aqueous methanol extract of the *O. baccatus*. This may provide more information on the nature of pharmacological preparations and the degree of variability in active chemical compounds among plants and surroundings, even within the same species.

MATERIALS AND METHODS

Experimental plant material collection

We conducted this study in the laboratories of the College of Science, King Saud University, during the period from 02/05/2024 to 15/01/2025. In May 2024, this study gathered the experimental plant *O. baccatus* from the Riyadh region, Kingdom of Saudi Arabia, located at latitude 24°97′35.7″N and longitude 46°46′33.6″E. A herbalist at King Saud University performed the classification of the plant.

Preparation of *O. baccatus* extract

Only the flowers and branches of *O. baccatus* were used in this study (Fig 1). Following ten to fifteen days of air drying in the shade, the plant samples were processed in a grinding machine to a fine powder. Then 80 g of *O. baccatus* (flowers and branches powdered) were submerged in 70% methanol for 48 hours at room temperature with shaking. Whatman No. 3 filter paper (Sigma, Germany) was used to filter the extract. The extract was then dried and concentrated using a rotary evaporator (Yamato RE300, Tokyo, Japan) at 40°C and lowered pressure (Khojali *et al*., 2023).

Infrared spectroscopy of *O. baccatus* extract

The *O. baccatus* extract was examined using FT-IR (Thermo Scientific, USA) and underwent a series of procedures to generate IR spectra. The scanning wavenumber range extended from 4000 to 500 cm^{-1}, with a resolution of 4 cm^{-1}. The spectral data were analyzed against references to identify the functional groups in the test samples. This made it possible to figure out what the IR spectra from the extract meant (Al-Shabib *et al*., 2018; Mabasa *et al*., 2021).

Gas chromatography-mass spectrometry (GC-MS) of *O. baccatus* extract

The extract of *O. baccatus* was analyzed *via* gas chromatography-mass spectrometry (Thermo Scientific, TSQ 8000 Evo; Waltham, MA, USA). Gas chromatography employing an Elite-5 mass spectrometer and a fused silica column was utilized to separate the components for analysis (Monika *et al*., 2022). The GCMS NIST (2008) library's database of known spectral components was utilized for comparative analysis.

Estimation of the *in-vitro* antioxidant activity

The evaluation of the antioxidant activity of *O. baccatus* extract was conducted *in vitro* through 2,22 -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical

Fig 1: Photo of *O. baccatus* which used in this study (Only the flowers and branches).

scavenging assays. The ABTS free radical scavenging assay was performed following the established protocol (Re *et al*., 1999). The concentrations of the samples varied from 15.625 to 500 µg/ml, measured in a disposable microcuvette with a path length of 1 cm. All evaluations were conducted in duplicate. The calculation of antioxidant activity was performed using the subsequent equation:

$$\%\text{Inhibition} = \frac{\text{A control-A sample}}{\text{A control}} \times 100$$

A control = Absorbance of negative control at the moment of solution preparation.
A sample = Absorbance of a sample after 5 min.

IC_{50} values were calculated from the graph illustrating the concentration of the sample required to scavenge 50% of the ABTS. The IC_{50} is used to denote the concentration of extracts necessary to neutralize 50% of free radicals. ABTS was quantified as mg GAE/L.

Estimation of the *in-vitro* antiparasitic activity

The experiment was conducted on the parasite *Eimeria intestinalis* that infects the intestines of rabbits. This parasite was obtained from the Parasitology Laboratory within the Department of Science at King Saud University.

Samples of *E. intestinalis* (sporulated oocyst) preserved in potassium dichromate solution (K_2, Cr_2, O_7) were subjected to cleaning with phosphate-buffered saline (PBS) According to the method (Murshed *et al*., 2024b). A suspension of the parasite was partitioned into seven segments. Each portion contained *O. baccatus* extract concentrations at 3.125, 6.25, 12.5, 25 and 50 mg/mL. A standard treatment of toltrazuril at a concentration of 30 µg/mL was employed for comparison, while potassium dichromate solution served as a negative control. Sporulated oocysts were assessed by documenting observations at 12, 24, 36, 48, 60 and 72 hours. Sample preparation was conducted using the McMaster Egg Counting Method (Long and Rowell, 1958). Slides were examined using a light microscope (PX51, Olympus Co., Tokyo, Japan) at a magnification of 10X.

Antiparasitic efficacy of each treatment was calculated using the following equation (Wang *et al*., 2009):

Antiparasitic efficacy (in %)=(B-T)/B × 100

Where,
B = Mean sporulated oocyst number of control.
T = Mean sporulated oocyst number of treatment.

Statistical Calculations

Data are presented as the mean ± SD derived from three independent observations. One-way ANOVA and Tukey's test ($p<0.05$ and $p<0.01$) were used to look for differences in the *in vitro* antioxidant and antiparasitic assays. A probability of $p<0.05$ was deemed significant and $p < 0.01$ was deemed very significant.

RESULTS AND DISCUSSION

FT-IR analysis of *O. baccatus* extract

The FT-IR analysis of the water-methanol extract derived from *O. baccatus* flowers and branches revealed the presence of 12 distinct compounds (Fig 2 and Table 1). The analysis revealed various characteristic peaks, each uniquely attributed to the presence of specific functional groups or phytochemical compounds. An analysis using FTIR spectrometry revealed principal bands ranging from 619 to 3409.48 cm^{-1}. The stretching and bending vibrations of N-H, C-H, C≡C, C=C, O-H, C-O, C-F and C-Br were observed across various bands, indicative of a diverse array of compound functionalities. A number of these are primary amines, alkanes, amine salts, alcohols, secondary alcohols, fluoro compounds, alkenes with methyl groups, monosubstituted alkynes and alkenes, halo compounds and alkanes with methyl groups (Jacox, 2003; Powell *et al*., 1966).

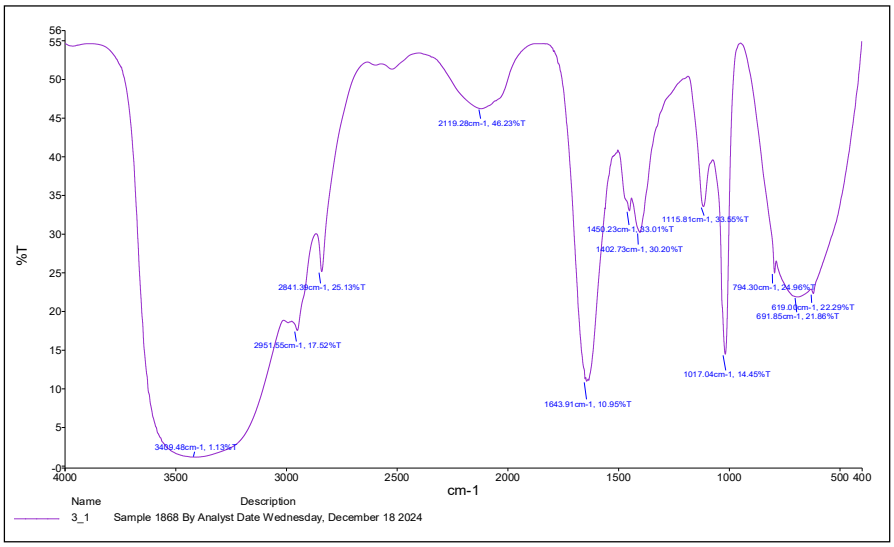

Fig 2: FT-IR chromatogram of *O. baccatus* aqueous methanol extract in methanolic medium showing the functional characteristic of the active chemical compounds.

GC-MS Analysis of *O. baccatus* extract

The GC-MS analysis of the aqueous methanol extract derived from *O. baccatus* flowers and branches identified around 11 key biologically active compounds (Table 2, Fig 3). 1-(2-piperidinyl)-2-propanone (18.33%), 2,3-dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one (9.64%), pyrrolidin-1-propionic acid (1.69%), 2-methoxy-4-vinylphenol (6.25%), octanal (0.94%), stevioside (4.34%), methyl 6-deoxy-alpha.-l-galactopyranoside (8.92%), tert-butyl acetoacetate (3.38%), l-gala-l-ido-octose (2.75%), hexadecanoic acid (7.26%) and (2r,3r)-2,3-epoxyoctadec-4-yn-1-ol (36.5%) were identified as the phytocompounds present in significant

Table 1: Analyze *O. baccatus* aqueous methanol extract to identify potential active chemical compounds using FT-IR.

Absorption (cm^{-1})	Transmittance (%)	Appearance	Group	Compound class
3409.48	1.13	Medium	N-H stretching	Primary amine
2951.55	17.52	Medium	C-H stretching	Alkane
2841.39	25.13	Strong	N-H stretching	Amine salt
2119.28	46.23	Weak	CÎC stretching	Alkyne/ monosubstituted
1643.91	10.95	Strong	C=C stretching	Alkene/monosubstituted
1450.23	33.01	Medium	C-H bending	Alkane/methyl group
1402.73	30.20	Medium	O-H bending	Alcohol
1115.81	30.55	Strong	C-O stretching	Secondary alcohol
1017.04	14.45	Strong	C-F stretching	Fluoro compound
794.30	24.96	Medium	C=C bending	Alkene/trisubstituted
691.85	21.86	Medium	C=C bending	Alkene/ disubstituted
619.00	22.29	Strong	C-Br stretching	Halo compound

Table 2: Analyze *O. baccatus* aqueous methanol extract Identification of phytochemical compounds by GC-Mass.

Retention time	Phytochemicals	Molecular formula	Molecular weight	Peak area%
6.97	1-(2-PIPERIDINYL)-2-PROPANONE	C8H15NO	141.21	18.33
8.2	2,3-DIHYDRO-3,5-DIHYDROXY-6-METHY L-4H-PYRAN-4-ONE	C6H8O4	144.12	9.64
9.89	PYRROLIDIN-1-PROPIONIC ACID	C7H13NO2	143.18	1.69
10.57	2-METHOXY-4-VINYLPHENOL	C9H10O2	150.17	6.25
12.01	OCTANAL	C8H16O	128.21	0.94
13.52	STEVIOSIDE	C38H60O18	804.9	4.34
15.54	METHYL 6-DEOXY-ALPHA.-L-GALACT OPYRANOSIDE	C7H14O5	178.18	8.92
17.74	TERT-BUTYL ACETOACETATE	C8H14O3	158.19	3.38
18.32	L-GALA-L-IDO-OCTOSE	C8H16O8	240.21	2.75
19.19	HEXADECANOIC ACID	C16H32O2	256.42	7.26
21.17	(2R,3R)-2,3-EPOXYOCTADEC-4-YN-1-OL	C8H12O2	142.16	36.5

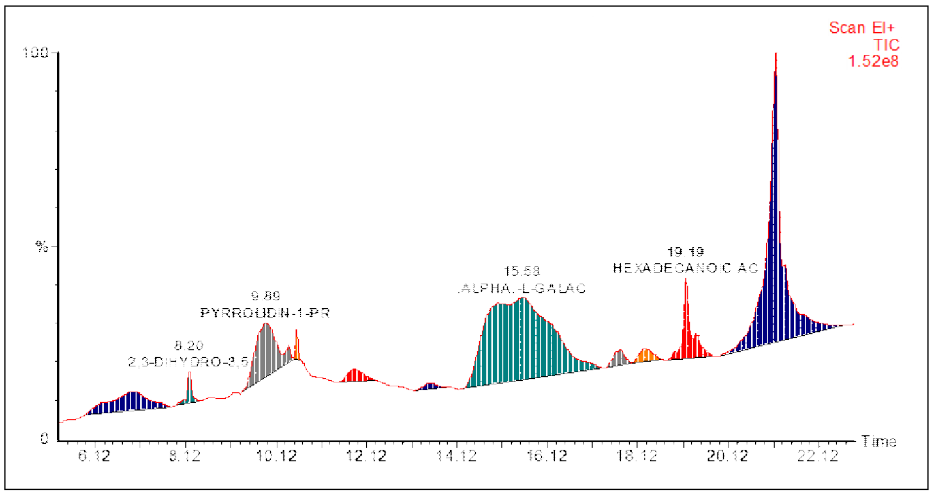

Fig 3: GC-MS phytochemical analysis of *O. baccatus* aqueous methanol Identification of phytochemical compounds.

quantities, while other compounds were detected in lesser amounts.

These results are similar to previous studies, but some compound concentrations differ, possibly due to plant environment. Phytochemical screening of the plant exhibited the presence of alkaloids, coumarins, saponins, fatty acids and steroidal compounds. The isolated phytoconstituents include Quercetin 3-O-p-coumaryl(1→6)-β-glucosyl(1→6) -β-glucoside-7O-α rhamnoside, Quercetin 3-O-β-glucosyl(1→2)-α-rhamnoside-7-O-α-rhamnoside, Quercetin 3-gentiobioside, Isoquercitrin, Quercitrin, Kaempferol glycosides, Rutin, Luteolin, Afzelin, Astragalin and phenols and fatty acids (Batanouny, 1981; Sarg *et al*., 1994a). It has high concentrations of glucosinolates also (Barakat *et al*., 1991; Sarg *et al*., 1994b). The LC-MS analysis of the methanolic extracts from the plant's roots and branches was then conducted, resulting in the identification of 8 and 13 major chemical constituents, respectively (Khojali *et al*., 2023).

Antioxidant activity *in vitro*

The radical scavenging activity of the aqueous methanol extract from the flowers and branches of *O. baccatus* was assessed using the ABTS scavenging assay. The extract was able to get rid of radicals, with an IC_{50} value of 147.032 µg/mL and inhibition rates ranging from 93.655% to 17.255% at 500 to 15.625 µg/mL. The ABTS scavenging assay showed statistically significant changes with the different concentrations of *O. baccatus* extract used (Table 3). This study discovered that the extract was more effective at stopping free radicals at the highest levels tested. The extract showed strong ABTS free radical scavenging activity and inhibition. These data indicate that *O. baccatus* functions as a natural antioxidant source.

These results are similar to previous studies with the difference in the inhibition rate, which may be due to the difference in the concentration of some active chemical compounds. Several studies have documented the ABTS scavenging activity of various sections of *O. baccatus*. The essential oils from the examined *O. arabicus* samples (flowers, leaves and stems) exhibited free-radical scavenging properties. The flowers demonstrated the highest efficacy, with an IC_{50} of 106.40 ± 0.19 µg/mL, followed by the leaves and stems, which had IC50 values of 143.80 ± 0.22 µg/mL and 159.60 ± 0.32 µg/mL, respectively. (Ullah *et al*., 2022). The plant extract has already been reported for its antioxidant impact, as stated by Al-Omar *et al*. (2020). The chemical components in a plant are responsible for addressing various ailments, including antioxidant potential (Sathiyamoorthy *et al*., 1999). Hassan *et al*. (2019) have shown that various factors such as edaphic, climatic and topographic factors influence the contents of plants. The quantity of the active ingredients may be affected due to the quality of water, as shown by Lv *et al*. (2021). Since the current study used different parts and expression units, it is difficult to directly compare the data with those reported in the literature.

In vitro* antiparasitic activity of *O. baccatus* against *E. intestinalis

The *in vitro* assessment of the efficacy of the extract against *E. intestinalis* showed that concentrations of 3.125, 6.25, 12.5, 25 and 50 mg/mL resulted in inhibition rates of 78%, 83%, 96%, 98% and 100%, respectively, during a 72-hour

Table 3: ABTS radical scavenging assay of different concentrations of phytochemicals isolated from the aqueous methanol extract of *O. baccatus.*

Concentration (µg/ml)	ABTS radical inhibition (%)	Concentration pair comparison	IC_{50} (µg/ml)
15.625 (T1)	17.255±0.2407	T1 vs T2**	
		T1 vs T3**	
31.25 (T2)	24.843±0.1685	T1 vs T4**	
		T1 vs T5**	
		T1 vs T5**	
62.5 (T3)	40.957±0.4352	T2 vs T3**	
		T2 vs T4**	
		T2 vs T5**	
125 (T4)	52.171±8.1644	T2 vs T6**	147.032± 1.5067
		T3 vs T4**	
		T3 vs T5**	
250 (T5)	81.390±0.8270	T3 vs T6**	
		T4 vs T4**	
		T4 vs T6**	
500 (T6)	93.655±0.6717	T5 vs T6**	

(*p≤0.05; **p≤0.01), shows significant differences compared between concentrations. Data are presented as mean ± SD, (n=3).

period. The results of antiparasitic efficacy demonstrated variability in sporulation and inhibition at various doses during an incubation time of up to 72 hours, with oocyst test results recorded every 12 hours. The results indicated the greatest inhibition of oocysts at concentrations of 50 mg/mL, 25 mg/mL and the reference drug, in contrast to lower concentrations where the inhibition rate diminished. Additionally, the inhibition increased with prolonged exposure time (Tables 4 to 9).

To our knowledge, no prior studies have elucidated the potential role of aqueous methanol extracts from *O. baccatus* in *E. intestinalis*. Consequently, this study was essential in elucidating this role. Studies indicate that *O. baccatus* is rich in various active compounds, with the methanolic extract derived from its branches demonstrating inhibitory effects on bacterial growth at minimum inhibitory concentration (MIC) values of 250 µg/mL, 15.6 µg/mL, 20 µg/mL and 500 µg/mL, respectively (Khojali *et al*., 2023). Previous studies

Table 4: (*In vitro* study) estimation of antiparasitic efficacy (in percent) of *O. baccatus* aqueous methanol extract against *E. intestinalis,* In 12 hours.

Treatments	Mean sporulated oocysts (B) in 12 hour	Antiparasitic efficacy (in%) = (B-T)×100/B in 12 hour
Control	197	0
T1 (3.125 mg/mL OB)	194	1.523^{a-c}
T2 (6.25 mg/mL OB)	190	3.553^{a-c}
T3 (12.5 mg/mL OB)	175	11.168^{a-c}
T4 (25 mg/mL OB)	168	14.721^{a-c}
T5 (50 mg/mL OB)	152	22.843^{a-c}
T6 (30 mg/mL Toltrazuril)	150	23.858^{a}

Table 5: (*In vitro* study) estimation of antiparasitic efficacy (in percent) of *O. baccatus* aqueous methanol extract against *E. intestinalis,* In 24 hours.

Treatments	Mean sporulated oocysts (B) in 24 hour	Antiparasitic efficacy (in%)=(B-T)×100/B in 36 hour
Control	198	0
T1 (3.125 mg/mL OB)	191	3.535^{a-c}
T2 (6.25 mg/mL OB)	185	6.566^{a-c}
T3 (12.5 mg/mL OB)	171	13.636^{a-c}
T4 (25 mg/mL OB)	151	23.737^{a-c}
T5 (50 mg/mL OB)	142	28.283^{a-c}
T6 (30 mg/mL Toltrazuril)	138	30.303^{a}

Table 6: (*In vitro* study) estimation of antiparasitic efficacy (in percent) of *O. baccatus* aqueous methanol extract against *E. intestinalis,* In 36 hours.

Treatments	Mean sporulated oocysts (B) in 36 hour	Antiparasitic efficacy (in%)=(B-T)×100/B in 36 hour
Control	196	0
T1 (3.125 mg/mL OB)	181	7.653^{a-c}
T2 (6.25 mg/mL OB)	170	13.265^{a-c}
T3 (12.5 mg/mL OB)	155	20.918^{a-c}
T4 (25 mg/mL OB)	132	32.653^{a-c}
T5 (50 mg/mL OB)	118	39.796^{a-c}
T6 (30 mg/mL Toltrazuril)	109	44.388^{a}

Table 7: (*In vitro* study) estimation of antiparasitic efficacy (in percent) of *O. baccatus* aqueous methanol extract against *E. intestinalis,* In 48 hours.

Treatments	Mean sporulated oocysts (B) in 48 hour	Antiparasitic efficacy (in%)=(B-T)×100/B in 48 hour
Control	196	0
T1 (3.125 mg/mL OB)	90	54.082^{a-c}
T2 (6.25 mg/mL OB)	73	62.755^{a-c}
T3 (12.5 mg/mL OB)	62	68.367^{a-c}
T4 (25 mg/mL OB)	51	73.980^{a-c}
T5 (50 mg/mL OB)	42	78.571^{a}
T6 (30 mg/mL Toltrazuril)	42	78.571^{a}

Table 8: (*In vitro* study) estimation of antiparasitic efficacy (in percent) of *O. baccatus* aqueous methanol extract against *E. intestinalis,* In 60 hours.

Treatments	Mean sporulated oocysts (B) in 60 hour	Antiparasitic efficacy (in%)=(B-T)×100/B in 60 hour
Control	196	0
T1 (3.125 mg/mL OB)	75	61.735^{a-c}
T2 (6.25 mg/mL OB)	51	73.980^{a-c}
T3 (12.5 mg/mL OB)	32	83.673^{a-c}
T4 (25 mg/mL OB)	21	89.286^{a-c}
T5 (50 mg/mL OB)	14	92.857^{a}
T6 (30 mg/mL Toltrazuril)	15	92.347^{a}

Table 9: (*In vitro* study) estimation of antiparasitic efficacy (in percent) of *O. baccatus* aqueous methanol extract against *E. intestinalis,* In 72 hours.

Treatments	Mean sporulated oocysts (B) in 72 hour	Antiparasitic efficacy (in%)=(B-T)×100/B in 72 hour
Control	196	0
T1 (3.125 mg/mL OB)	42	78.571^{a-c}
T2 (6.25 mg/mL OB)	32	83.673^{a-c}
T3 (12.5 mg/mL OB)	8	95.918^{a-c}
T4 (25 mg/mL OB)	3	98.469^{a-c}
T5 (50 mg/mL OB)	0	100^{a}
T6 (30 mg/mL Toltrazuril)	0	100^{a}

Notes: Means with distinct superscripts within a column are significantly different, (a) Significant differences Concentration of (OB) and reference treatment vs. control $p < 0.01$, (ab) Significant differences were minor Concentration of (OB) and reference treatment vs. control $p < 0.05$, (c) Significant differences Concentration of (OB) vs. reference treatment $p < 0.01$, (cd) Significant differences were minor Concentration of (OB) vs. reference treatment $p < 0.05$.

OB: Means *O. baccatus*

have also reported similar results (Kaithwas *et al*., 2011; Khan *et al*., 2019). Because of its extraordinarily high glucosinolate content, this plant's nematicidal action against the root-knot worm *Meloidogyne javanica* was assessed. 100% of second-stage juveniles were immobilized in *in vitro* tests using plant aqueous extracts after 48 hours of exposure to 4% root-core extract; 8% root-core extract inhibited their hatching by 87%, whereas stem, flower and root bark showed reduced activity (Oka *et al*., 2014). Strong growth suppression (over 96%) of the malaria parasite *Plasmodium falciparum* was shown by *O. baccatus* (Sathiyamoorthy *et al*., 1999). The ethanolic extract of the plant has also been shown to have anti-inflammatory and anti-free radical activities. Also shown to fight parasites and helminths (Alqasoumi *et al*., 2012).

CONCLUSION

Habitat profoundly affects the synthesis and diversity of plant secondary metabolites; different geographical and ecological harvesting zones produce various chemical compositions, even within the same plant species. Extracts from the flowers and branches of *O. baccatus* include many active chemical and antioxidant constituents with antiparasitic effects. *O. baccatus* is renowned for its substantial impact on antiparasitic properties. The findings of this study indicate that the extract of flowers and branches from *O. baccatus* exhibits potential oocysticidal characteristics, which might be utilized in the treatment of coccidiosis. This study provides a significant basis for employing *O. baccatus* solution in the treatment of Eimeria sp. in rabbits. More research needs to be done to find out how well the extract works in living tissues and how the molecular processes that control its action work.

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Disclaimers

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Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or

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