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RESEARCH ARTICLE

In vitro Anticoccidial, Antioxidant Activities and Biochemical Screening of Methanolic and Aqueous Leaves Extracts of Selected Plants

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ABSTRACT

Avian coccidiosis, a protozoan parasitic disease caused by genus *Eimeria*. Due to emergence of drug resistant *Eimeria* species, this study was aimed to evaluate anticoccidial potentials of *Ficus racemosa*, *Cassia fistula* and *Syzygium cumini* leaves extracts. In vitro anticoccidial efficacy of extracts was evaluated by oocysts sporulation inhibition and sporozoites viability inhibition assays of mixed *Eimeria* species oocysts. The set up was examined after 48hrs of incubation. DPPH radical scavenging activity, ferric reducing antioxidant power and total antioxidant capacity were used for the evaluation of antioxidant potential of extracts. Among tested extracts maximum oocysts sporulation inhibition 86.81±2.35% and sporozoites viability inhibition was 86.73±1.67% at concentration 30 mg/ml of *C. fistula* methanolic leaves extract against *E. mitis* and *E. tenella* respectively. Highest radical scavenging capacity 67.82±0.00 and reducing power 2.17±0.01 was shown by *F. racemosa* and *C. fistula* methanolic leaves extract respectively. Maximum total antioxidant power was observed in *C. fistula* 30.95±0.35 and *F. racemosa* 21.93±1.41µg/mg methanolic leaves extracts. Antioxidant compounds including phenols, flavonoids, alkaloids, saponins, carbohydrates *etc.* were detected through biochemical screening of selected plants extracts. The maximum amount of phenols 32.50±0.00µg/ml and flavonoids 40.00±1.00µg/ml were recorded in *C. fistula* methanolic extracts. It is concluded that selected plants methanolic extracts possess best anticoccidial and antioxidant activities due to presence of medicinally important phytochemicals. Further research is needed for identification and isolation of anticoccidial active compounds from these plants that can be used in the formulation of drugs against coccidiosis.

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INTRODUCTION

Coccidiosis, an avian, parasitic disease caused by different species of *Eimeria* (Abbas *et al*., 2015). It is characterized by bloody diarrhea, decrease in weight and high mortality. Priced anti coccidial drugs and high mortality rate cause 240 million dollars loss in Java and US$ 0.005 in Pakistan (Rashid *et al*., 2019; Pawestri *et al*., 2020). Most illnesses, including coccidiosis, are primarily associated with oxidative stress induced by free radicals. The currently available treatments and vaccinations for control of coccidiosis are costly and chemicals are producing resistance against its parasites (khater *et al*., 2020) therefore, there is a need for exploring new agents such as plants for drug formulation against *Eimeria* species (Abbas *et al*., 2019a). In the current study the crude methanolic and aqueous leaves extracts of three plants *Ficus racemosa*, *Cassia fistula* and *Syzygium cumini* were evaluated for their antioxidant capacity against coccidiosis. The *F. racemosa* (Moraceae) a well-known medicinal plant rich in phenolics and flavonoid exhibit strong antioxidant and antimicrobial activities (Bagyalakshmi *et al*., 2019). *C. fistula* (leguminosae) commonly known as amaltas, possess medicinally important phytochemicals (Kamath *et al*., 2019). *S. cumini* (Myrtaceae) is widely distributed in tropical and subtropical areas, commonly known as Jamun having a wide range of therapeutic characteristics (Eshwarappa *et al*., 2014). This work was aimed to investigate anticoccidial, antioxidant activities and

biochemical screening of crude methanolic and aqueous leaves extracts of selected plants since no previous studies recorded on anticoccidial effects of the plants selected in this study. The present studies will have a great significance in evaluating these plants as a potential source of drug formulation for coccidiosis.

MATERIALS AND METHODS

Plants collection, identification and extraction: Fresh leaves of *F. racemosa*, *C. fistula* and *S. cumini* were collected in June to September 2018 from the locality of Quaid-i-Azam university (QAU), Islamabad, Pakistan.

Identification of plants were done through flora of Pakistan and taxonomist (Abdullah *et al*., 2018). Plants specimen were deposited in herbarium of Pakistan, Quaid-i-Azam University under voucher code of 130863 for *F. racemosa*, 130862 for *C. fistula* and 130861 for *S. cumini*. Collected leaves were washed thoroughly, shade dried and grounded to powder. The leaves powder was stored at room temperature in airtight containers until for use.

Methanolic and aqueous extracts of *F. racemosa*, *C. fistula* and *S. cumini* leaves powder were obtained by using maceration method of extraction (Cedric *et al*., 2018). Extract yield was calculated in grams and expressed as percentage (Gahlot *et al*., 2018).

In vitro anticoccidial activities

Samples collection and identification of *Eimeria* species: Coccidiosis suspected chicks guts were collected from veterinary research institute (VRI) Peshawar. By microscopic conformations, gut contents containing *Eimeria* species oocysts were collected and stored in 2.5% $K_2Cr_2O_7$ solution (Wajiha *et al*., 2018).On the basis of oocysts morphology, size and site of infection in gut different species of *Eimeria* were identified (Gadelhaq *et al*., 2018).

Oocysts sporulation inhibition assay: In vitro sporulation inhibition effect of extracts were evaluated in petri dishes. In each petri dish total volume of 2ml of each concentration of the extracts (2.5, 5, 10, 20 and 30 mg/ml),1500 non sporulated oocysts/ml were inoculated and incubated at 28°C for 48hrs. The number of un-sporulated and sporulated oocysts were counted in each petri dish and sporulation percentage was calculated by counting the number of sporulated oocysts in a total of 100 oocysts. DMSO as a negative and amprolium (1.25mg/ml) as a positive control were used. The following formula was used for the calculation of sporulation inhibitory percentage (Cedric *et al*., 2018).

$$Sporulation\ Inhibitory\ Percentage\ (SP\%) = \frac{SP\%\ of\ control\ \_\ SP\%\ of\ extract}{SP\%\ of\ control} \times 100$$

Sporozoites viability inhibition assay: Sodium hypochlorite (30%v/v) was added to sporulated oocysts and centrifuged for 10 minutes at 600g. Sporulated oocysts present in supernatant was collected. For excystation, sporulated oocysts stored in $K_2Cr_2O_7$ were washed several times with HBSS (PH 7.2). Then 125ml HBSS, 0.32g trypsin, 0.25g bile salt was added and incubated. After incubation, centrifuged for 10minutes at 3,000-5,000xg. Liberated sporozoites were collected and washed with HBSS. For the evaluation of antisporozoite effects of selected plants crude extracts, to the 2ml of each concentration (2.5, 5, 10, 20 and 30 mg/ml) of different extracts, 1500 sporozoites/ml was added. DMSO as a negative and amprolium (1.25mg/ml) as a positive control were used. The complete set up was examined for the viability of sporozoites after 48hrs. viability percentage of sporozoites was determined by counting the number of viable sporozoites in total of 100 sporozoites. The following formula was used for determination of viability inhibitory percentage (Cedric *et al*., 2018).

$$Viability\ Inhibition\ Percentage\ (Vi\%) = \frac{Vi\ \%\ of\ control\ \_\ Vi\ \%\ of\ extract}{Vi\%\ of\ control} \times 100$$

Antioxidant assays

DPPH radical activity: For the determination of free radical scavenging activity, the method of Tai *et al*. (2011) with minor modifications was used. In 96 well plates different concentrations of extract (5, 10, 15 and 20 µg/ml) were taken. DPPH was added to the all rows for obtaining 200µl final concentrations. DMSO and ascorbic acid were taken as negative and positive control respectively. Absorbance was measure by micro plate reader (Platos R 496) at 630 nm after 1 hour of incubation. The following formula was used for the calculation of radical scavenging percentage.

$$Radical\ Scavenging\ (\%) = [\frac{(A0 - A1)}{A0} \times 100]$$

IC_{50} values were calculated for antioxidant activity of each sample.

Reducing power estimation: Selected plants crude extracts reducing power was determined according to the standard procedure (Patil *et al*., 2009). Phosphate buffer and potassium ferricyanide was mixed with different concentrations of extract. After incubation of mixture 10% of tri chloroacetic acid (2.5ml) was added. After centrifugation distilled water (2.5ml) and 0.1% ferric chloride (0.5ml) was added to supernatant. By using spectrophotometer absorbance was measured at 700nm.

Total antioxidant capacity estimation: Phospho molybdenum method was used for the evaluation of total antioxidant capacity (Sakat *et al*., 2010).180µl of phospho molybdenum reagent is mixed with 20µl of test samples (4mg/ml). After incubation samples were transferred to 96 well plates and cooled at room temperature. DMSO as negative and ascorbic acid as positive control were taken. Absorbance was measured at 630 nm by using micro plate reader (Platos R 496).

Biochemical screening

Qualitative analysis: The crude extract was screened using conventional techniques for the existence of bioactive compounds. Different chemical tests on crude extracts were performed to distinguish different components like phenols, flavonoids, carbohydrates etc. using standard methods (Sofowra, 1993).

Quantitative analysis

Determination of total flavonoid contents (TFC): The colorimetric technique of aluminum trichloride ($AlCl_3$) was used to determine the total flavonoids contents with slight changes according to system suitability (Kaneria *et al*., 2014). Quercetin were taken as control. By using micro plate reader absorbance was measured at 405 nm.

Determination of total phenolic contents (TPC): Total phenolic contents were determined by using Folin Ciocalteu reagent technique with slight modifications (Jagadish *et al*., 2009). The absorbance was measured with a UV spectrophotometer at a steady wavelength of 750 nm. All the tests were performed in triplicates and data were presented as Mean ± SD.

Statistical analysis: The obtained data were analyzed statistically through SPSS, version 23. The triplicate data were expressed as Mean ± SD. Waller-Duncan test is used for comparison of values. Significance level was considered at $P<0.05$.

RESULTS

Percentage yield: In present study from 150g of leaves powder of each selected plant, aqueous and methanolic extracts were prepared. The percentage yield of methanolic extract of *F. racemosa* was the highest (4.2%) followed by *C. fistula* (3.76%) and *S. cumini* (2.15%) than their corresponding aqueous extracts (Table 1).

In vitro Anticoccidial activities: The three different *Eimeria* species *E. tenella* (47%), *E. necatrix* (29%) and *E. mitis* (24%) were identified.

Oocysts sporulation inhibition assay: In vitro oocysts sporulation inhibition activity of different extracts of experimental plants against *Eimeria* species oocysts were evaluated. Maximum oocysts sporulated in negative control group as their sporulation inhibition efficacy is lowest (2.20±1.31%). Among extracts, highest sporulation inhibition efficacy was (86.81±2.35%) at concentration 30 mg/ml of methanolic extract of *C. fistula* against *E. mitis.* In contrast the lowest efficacy was (6.11±2.54%) at concentration 2.5mg/ml of aqueous extract of *F. racemosa* against *E. necatrix* (Fig. 1, 2).

Sporozoites viability inhibition assay: In present study among extracts, highest sporozoites viability inhibition was (86.73±1.67%) at concentration 30 mg/ml of *C. fistula* methanolic leaves extracts against *E. tenella*. The lowest efficacy was (2.11±3.62%) at concentration 2.5 mg/ml of *S. cumini* aqueous extract against *E. necatrix* (Fig. 3).

Antioxidant assays

DPPH radical activity: Selected plants extracts free radical scavenging was evaluated by DPPH radical assay. A change from purple colour to yellow was observed. The results revealed that maximum radical scavenging activity was observed in *F. racemosa* (67.82±0.00). The lowest IC_{50} values of *F. racemosa* exhibited the highest radical scavenging activity. While aqueous extracts of selected plants showed lowest inhibition at highest IC_{50} values (Table 2).

Ferric reducing power: The experimented plants extract exhibit increase in reducing power as the concentration of extract increased. Methanolic leaves extract of *C. fistula* showed highest reducing power (2.17±0.01) at 600 µg/ml concentration similar to the reducing power of ascorbic acid (2.88±0.06) at the same concentration (Table 3).

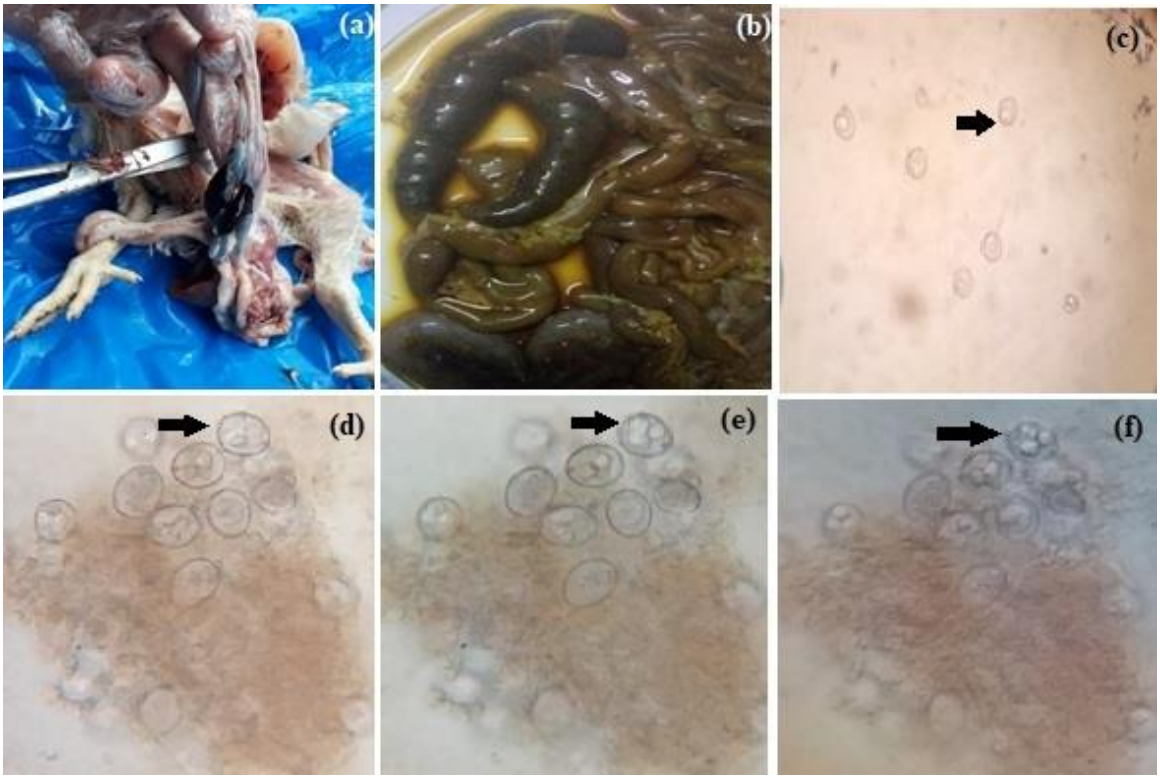

Fig: 1: (a) Coccidiosis infected chick **(b)** Caecum and intestine of infected chick **(c)** un sporulated oocysts **(d), (e)** and **(f)** Sporulated oocysts.

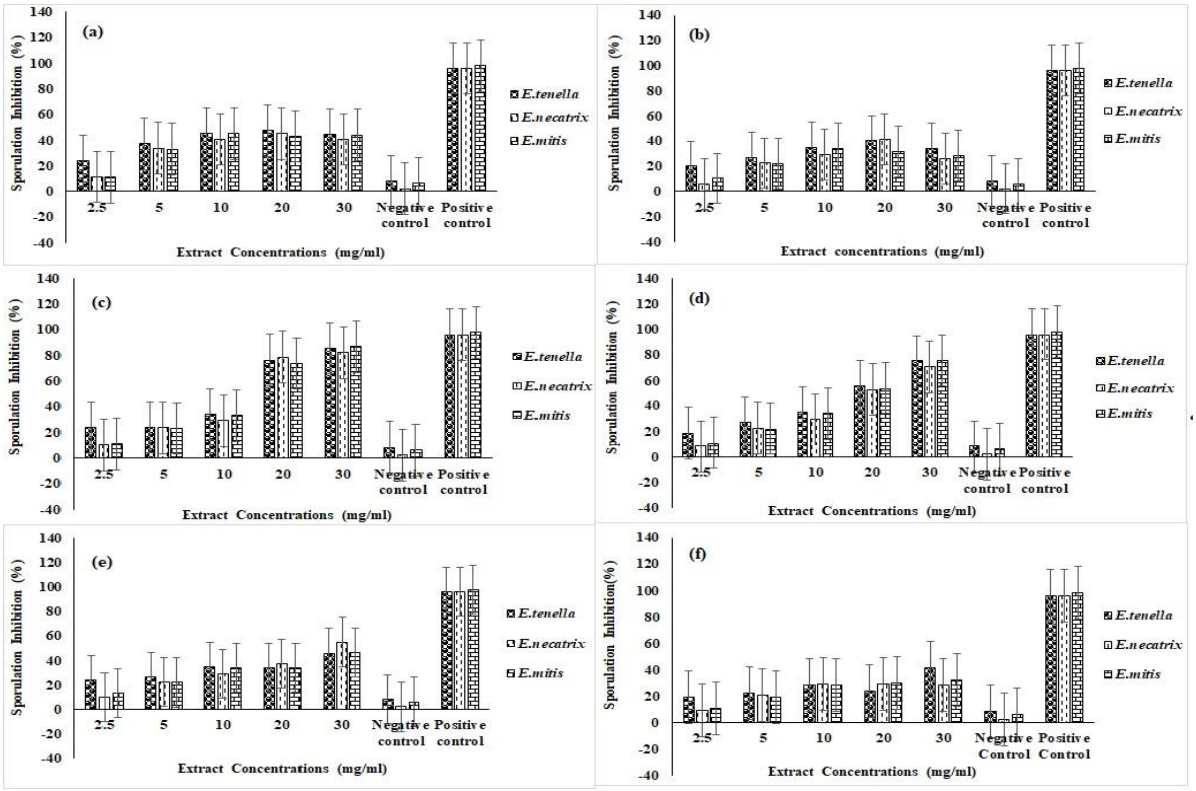

Fig 2: (a), (b): Effect of *F. racemosa* methanolic and aqueous leaves extracts different concentration on sporulation inhibition of different *Eimeria* species oocysts. (**c), (d).** Effect of *C. fistula* methanolic and aqueous leaves extracts different concentration on sporulation inhibition of different *Eimeria* species oocysts. **(e), (f).** Effect of *S. cumini* methanolic and aqueous leaves extracts different concentration on sporulation inhibition of different *Eimeria* species oocysts.

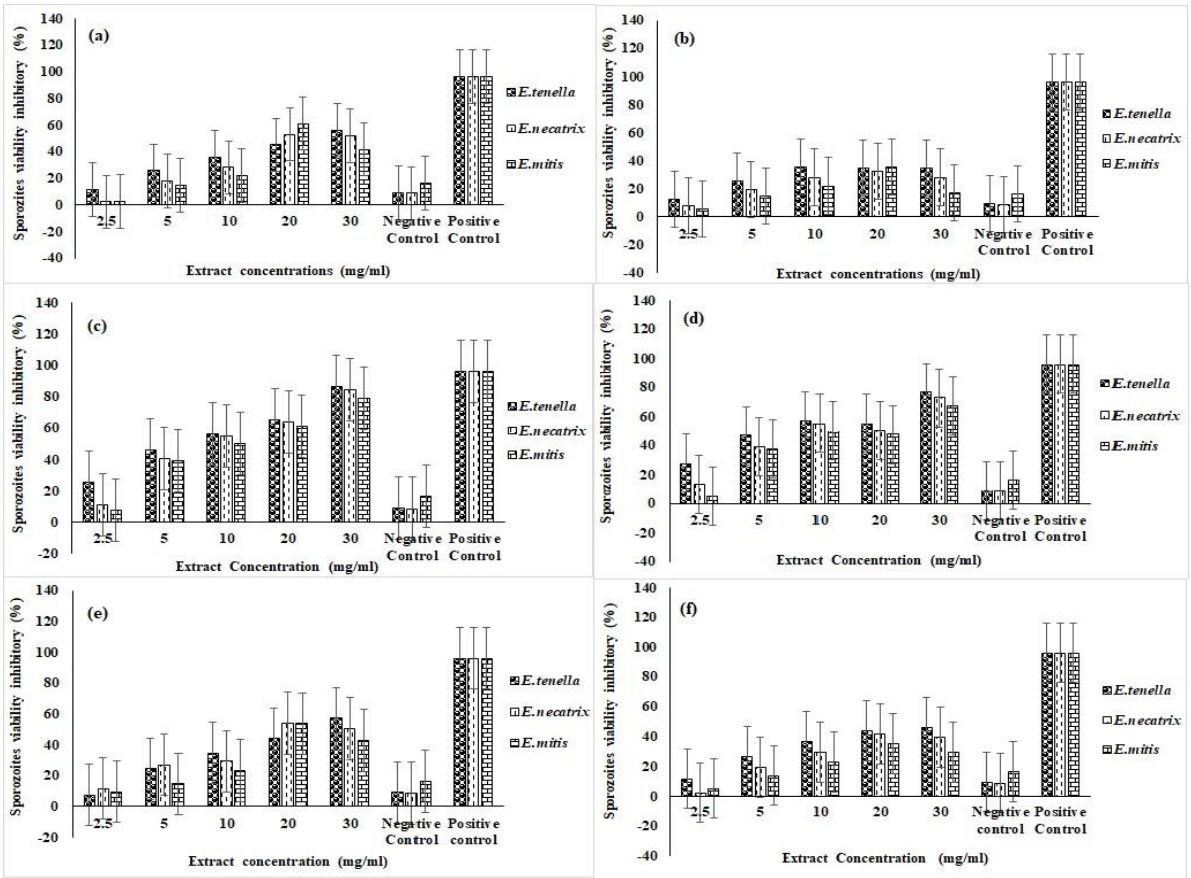

Fig 3: (a), (b): Effect of *F. racemosa* methanolic and aqueous leaves extracts different concentration on sporozoites viability inhibition of different *Eimeria* species. **(c), (d).** Effect of *C. fistula* methanolic and aqueous leaves extracts different concentration on sporozoites viability inhibition of different *Eimeria* species. **(e), (f).** Effect of *S. cumini* methanolic and aqueous leaves extracts different concentration on sporozoites viability inhibition of different *Eimeria* species.

Table 1: Percentage yield of the selected plants extracts

Plants	Solvents	Weight of the plant powder (g)	Weight of the crude extract obtained (g)	Percentage of Yield %
Ficus racemosa	Methanol	150	6.31	4.2
	Water	150	3.99	2.66
Cassia fistula	Methanol	150	5.65	3.76
	Water	150	4.87	3.24
Syzygium cumini	Methanol	150	3.23	2.15
	Water	150	2.54	1.69

Table 2: DPPH radical scavenging activity (%) and IC_{50} values of different extracts of selected plants

Parameters		*Ficus racemosa*	*Cassia fistula*	*Syzygium cumini*
Conc. µg/ml	5	67.82±0.00^{b}	42.91±1.98^{a}	57.10±2.37^{a,b}
	10	50.34±1.52^{a}	49.10±2.33^{a}	56.84±2.45^{a}
	15	51.03±0.29^{a}	28.38±1.29^{b}	56.32±1.94^{a}
	20	48.35±0.00^{l}	56.36±0.62^{a}	43.86±0.63^{a}
	Ascorbic acid	79.29±2.34^{b}	70.45±5.24^{b}	72.35±1.82^{b}
Solvents	Methanol	52.82±3.75^{a}	36.96±1.71^{a}	40.16±1.67^{a}
	Aqueous	52.82±0.21^{a}	33.74±6.02^{a}	36.62±2.56^{a}
IC_{50} values µg/ml	Methanol	5.18	6.93	78.31
	Aqueous	23.22	69.97	7.19
	Ascorbic acid	1.95	6.55	2.53

Means in the same row and sub table not sharing the same superscripts are significantly different at p<0.05 in the two sided test of equality for column means.

Table 3: Total reducing power of different solvents extracts of selected plants

Conc. µg/ml	Solvents	Absorbance of extracts and standard (Mean ± SD)			
		Ficus racemosa	*Cassia fistula*	*Syzygium cumini*	Ascorbic acid
100	Methanol	0.07±0.00^{ij}	0.81±0.00^{f}	0.07±0.00^{ij}	0.92±0.01
	Aqueous	0.02±0.01^{j}	0.13±0.06^{hi}	0.07±0.00^{e}	
300	Methanol	0.23±0.02^{h}	1.22±0.02^{d}	0.23±0.02^{h}	1.36±0.03
	Aqueous	0.04±0.01^{ij}	1.26±0.09^{d}	0.23±0.02^{c}	
600	Methanol	0.53±0.02^{g}	2.17±0.01^{b}	0.53±0.02^{g}	2.88±0.06
	Aqueous	0.05±0.02^{ij}	2.16±0.02^{b}	0.53±0.02^{a}	

Same superscript letter values are not significantly different at P≥0.05.

Table 4: Total antioxidant capacity of different solvents extracts of selected plants

Conc. (µg/ml)	Solvents	Absorbance of extracts (Mean ± SD)		
		Ficus racemosa	*Cassia fistula*	*Syzygium cumini*
1000	Methanol	21.93±1.41^{bc}	30.95±0.35^{a}	19.53±2.14^{c}
	Aqueous	20.53±1.48^{c}	26.84±2.68^{ab}	11.32±2.07^{d}

Same superscript letter values are not significantly different at P≥0.05.

Total anti-oxidant capacity: In total antioxidant capacity estimation Mo (VI) is converted into Mo (V) by the antioxidants of extracts. By estimating total antioxidant capacity, it was observed that methanolic leaves extracts of *C. fistula* (30.95±0.35µg/mg) and *F. racemosa* (21.93±1.41µg/mg) possess maximum antioxidant activity. The antioxidant power of *F. racemosa* was in the range of standard ascorbic acid (Table 4).

Biochemical screening

Qualitative analysis: Different biochemical tests were used for the qualitative biochemical screening of all extracts. Results of biochemical tests showed that methanolic leaves extracts of all the selected plants are more rich in antioxidant compounds as compared to their aqueous extracts. Flavonoids were extracted from almost all the plants but in the most abundant were found in *C. fistula* methanolic extracts. Few compounds like proteins and alkaloids were also observed in aqueous but not in their corresponding methanolic extracts (Table 5).

Quantitative analysis: Amongst the different solvents and plants extracts, maximum total phenolic (32.50±0.00 µg/ml) and flavonoids (40.00±1.00µg/ml) contents were in methanolic extract of *C. fistula*, while least amount of total phenolic contents were in aqueous extract of *F. racemosa* (25.00±1.73µg/ml) and *S. cumini* (21.90±1.73 µg/ml). Total flavonoids in the lowest content were observed in aqueous extract of *F. racemosa* (26.67±0.58 µg/ml) (Table 6).

DISCUSSION

Coccidiosis is an avian disease caused by *Eimeria* parasite infects poultry causing huge economic losses. Effective, alternative herbal therapies for control of avian coccidiosis arises due to coccidiostats resistance in *Eimeria* species (Abbas *et al*., 2019b). Therefore, the current study was aimed to explore anticoccidial and antioxidant activities of selected plants. Results of the present study revealed that highest percentage yield was of methanolic leaves extracts of experimental plants. In agreement with present work, Truong *et al*. (2019) reported the highest extraction yield of methanol as compared to other solvents. Specifying that strong polar solvents favors the efficiency of extraction. It was observed in the present investigations that anticoccidial potency and sporulation inhibition percentage was directly proportional to concentration of methanolic leaves extracts. Similarly, Desalegn and Ahmed (2020) proposed that aloe species anticoccidial effect was increased with increasing concentrations. However, Cedric *et al*. (2018) observed maximum in vitro sporulation inhibition of *Eimeria* oocysts with aqueous extracts of *Psidium guajava*. Plants having antioxidant potentials are harmful for parasites like *Eimeria* species by their reaction with free radicals and production of oxidative stress (Idris *et al*., 2017).

Selected plants particular mechanism of action is unknown but from the results of the current study it can be anticipated that their anti sporulation effects may be due to the interference of antioxidant phytochemicals in physiological process of sporulation like disruption in O_2 consumption and inactivation of enzymes important for

Table 5: Preliminary qualitative biochemical analysis of selected plants crude extracts

S.N	Chemical compounds	Test name	Observations	*Ficus racemosa*		*Cassia fistula*		*Syzygium cumini*	
				Methanol	Water	Methanol	Water	Methanol	Water
1	Alkaloids	Mayer's test	Pale ppt formed	–	+	+	+	+	+
2	Saponins	Froth test	Stable persistent	+	–	+	+	+++	++
3	Carbohydrates	Fehling test	Brick red ppt	-	–	–	–	+	+
4	Phenols and tannins	Ferric chloride	Bluish color formed	+	+	+	–	+++	–
5	Flavaniods	Alkaline reagent test	Reddish pink colors	+	+	+	+	+	+
6	Terpenoids	Salkowaski test	Reddish brown coloration	+	+	+	+	+	+
7	Glycosides	Liebermann's test	Blue and green coloration	-	-	+	+	+	+
8	Proteins	Millon's test	Red precipitate	+	+	–	+	+	+

(+) = Presence, (-) = Absence, (++) = Moderate concentration, (+++) = High Concentration.

Table 6: Quantitative phytochemical screening (Total phenolic contents and Total flavonoids contents) of selected plants crude extracts

Sr. No	Plants	Solvents	Total phenolic contents(µg/ml)	Total flavonoids contents (µg/ml)
1	*Ficus*	Methanol	28.33±2.08^{ab}	32.57±2.00^{ab}
	racemosa	Aqueous	25.37±1.73^{ab}	26.76±0.58^{b}
2	*Cassia*	Methanol	32.50±0.00^{a}	40.00±1.00^{a}
	fistula	Aqueous	25.00±1.73^{ab}	32.63±2.00^{ab}
3	*Syzygium*	Methanol	26.78±1.73^{ab}	30.73±1.15^{ab}
	cumini	Aqueous	21.90±1.73^{b}	29.20±1.15^{b}

Same superscript letter values are not significantly different at P≥0.05.

sporulation as stated by Desalegn and Ahmed (2020). Sapnonin, a phytochemical present in these plants may kill the parasite by acting on their cell membrane cholesterol. These extracts may affect cytoplasmic components of oocysts and exhibit concentration dependent inhibition of coccidia sporozoites viability (Cedric *et al*., 2018; Lopez *et al*., 2019). Osmotic effects of extracts on sporozoites may cause their mortality or blockage of calcium channels receptors may lead to disruption of Ca^{2+} signaling necessary for sporozoites (Sarkozi *et al*., 2007).

High radical scavenging activity of methanolic extract of *C. fistula* in present study are in favors with the previous study reported by Deeksha and Arunachalam (2019). During determination of the reducing power of extracts, Fe^{3+} is converted into Fe^{2+}, Fe^{2+} then reacts $FeCl_2$ and results the formation of complex ferrous. Like present study, Kifayatullah *et al*. (2015) reported that ethanolic extracts of *Pericampylus glaucus* (Lamk) exhibit increase in reducing power as the concentration of the extract increased. According to the Eshwarappa *et al*. (2014), *S. cumini* methanol extract had better reducing power and possessed equal potential with the standard ascorbic acid used. In Phospho molybdenum method of TAC estimation, through antioxidant mediators present in extract green colour phosphate/Mo (V) complex formation occurred. Methanolic leaves extracts of *F. racemosa* and *C. fistula* possess maximum antioxidant power. Munira *et al*. (2018) reported that total antioxidant activity of *F. racemosa* methanolic leaves extract is in the range of standard catechin. Various bioactive compounds like phenols, flavonoids, alkaloids, saponins, terpenoids *etc* commonly used as medicinal attributes were confirmed in all selected plants. In *F. raecmosa* extracts qualitative phytochemical screening confirmed tannins, alkaloids, flavonoids, saponins etc. (Bagyalakshmi *et al*., 2019). Pavai *et al*. (2019) confirmed the presence of different phytochemicals like saponins, carbohydrates, alkaloids, phenols and etc in *C. fistula* extracts. *S. cumini* leaves extracts contained different medicinally important phytochemicals like phenols, tannins, saponins, proteins etc. (Ramos and Bandiola, 2019) which is in accordance to present study. Among all the tested extracts, maximum total phenolic and flavonoids contents were found in methaolic extract of *C. fistula*. Deeksha and Arunachalam, (2019) also reported maximum TP and TF contents in *C. fistula* extract. In contrast the work of Sumi *et al*. (2016) reported that *F. racemosa* methanolic leave extract contained a significant amount of phenolic (20.2mgQE/g) and flavonoid (22.81mgQE/g) contents. According to Kaneria *et al*. (2013) antioxidant activities of extracts are highly related to their total phenols and flavonoids contents.

Conclusions: The present findings showed that the methanolic extracts of selected plants have significant anticoccidial and antioxidant activities due to presence of medicinally important phytochemicals. These biochemicals can be considered as best substitutes to chemical anticoccidials. The current preliminary evaluation is significantly important for isolation and identification of anticoccidial compounds from *F. racemosa*, *C. fistula* and *S. cumini* plants applying column chromatography, HPLC or GC-MS.

Authors contribution: Both authors contribute substantially to this manuscript.

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