Evaluation of *Ficus racemosa* (Moraceae), *Cassia fistula* (Fabaceae) and *Syzygium cumini* (Myrtaceae) as a potential source of anticoccidial agents

By

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Department of Zoology

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

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Evaluation of *Ficus racemosa* (Moraceae), *Cassia fistula* (Fabaceae) and *Syzygium cumini* (Myrtaceae) as a potential source of anticoccidial agents

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By

Wajiha

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2022

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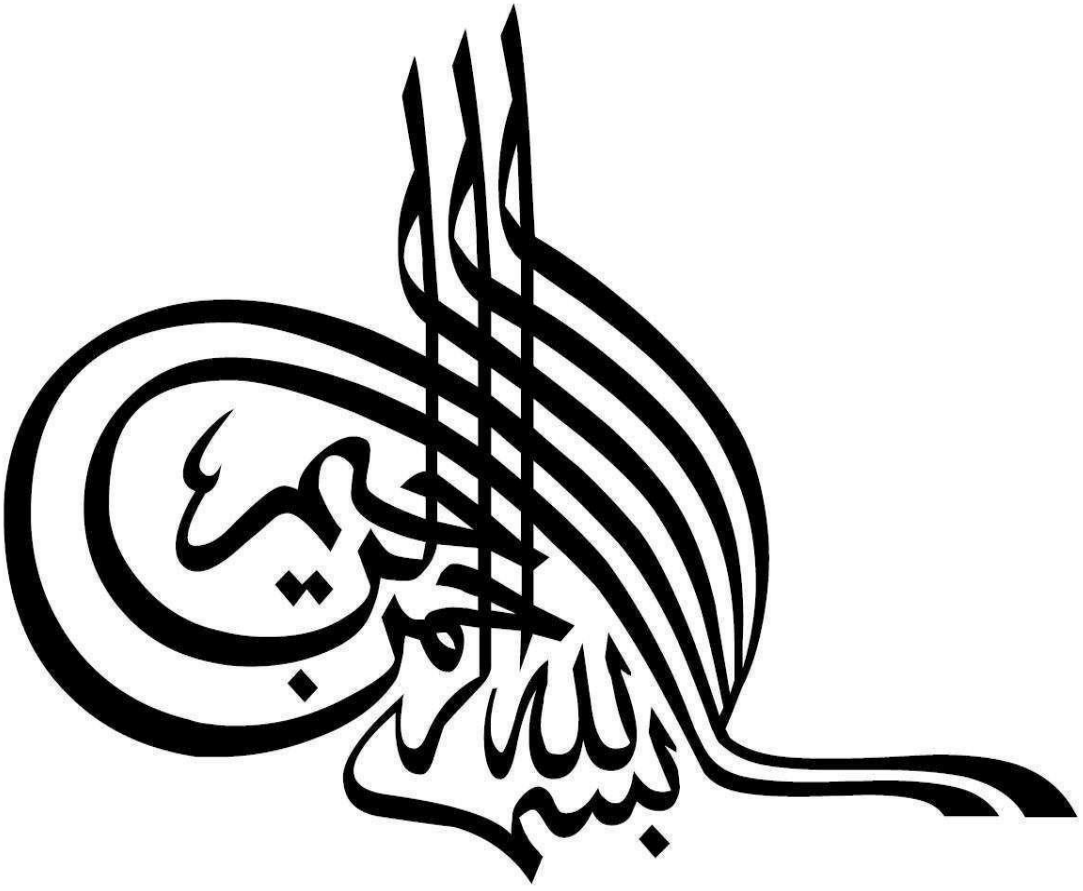

In the name of Allah,
the Most Beneficent,
the Most Merciful

Dedicated to my Parents

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GENERAL ABSTRACT

Coccidiosis has a serious impact on the poultry business, which is the most economically significant sector in the world. The causative agent of the avian coccidiosis is protozoan parasite of the genus *Eimeria*. This lethal disease caused economic loss of almost $3 billion worldwide. Due to development of resistance against available drugs and vaccines there is need of alternate strategy for the control. Therefore, this study's main goal was to evaluate the anticoccidial and antioxidant properties of the crude methanolic and aqueous leaf extract of *Syzygium cumini*, *Ficus racemosa*, and *Cassia fistula*.

Coccidiosis suspected samples were collected from Veterinary Research Institute (VRI), Peshawar. Different species of *Eimeria* were identified on the basis of oocyst morphology, size and site of infection in gut. The *in vitro* anticoccidial activity of extracts different concentration was evaluated using different assays on *Eimeria* spp oocysts that inhibit sporozoite viability and oocyst sporulation. After 48 hours of incubation, the setup was examined. The antioxidant potential of the extracts was evaluated by total antioxidant capacity estimation, ferric reducing antioxidant power and DPPH radical scavenging assay. On phytochemical screening, anti-oxidant components such as carbohydrates, phenols, saponins, alkaloids, flavonoids etc were found in different plant extracts. *C. fistula* methanolic leaf extract showed maximum oocyst sporulation inhibition (86.81±2.35%) and sporozoites viability inhibition (86.73±1.67) against *E. mitis* and *E. tenella*, respectively, at a concentration of 30mg/ml. *C.fistula* and *F. racemosa* methanolic leaf extract had the greatest reduction power (2.17±0.01) and DPPH radical scavenging capacity (67.82±0.00). The highest levels of total antioxidant activity were found in the methanolic leaf extracts of *C. fistula* (30.95±0.35) and *F. racemosa* (21.93±1.41). Maximum flavonoids (40.00±1.00) and phenols (32.50±0.00) g/ml were found in the methanolic extracts of *C. fistula*. It is concluded that because of the medicinally significant phytochemicals, certain plant methanolic extracts have the greatest anticoccidial and antioxidant activity.

The methanolic leaves extract of selected plants were fractionated by column chromatography and thin layer chromatography. Preliminary quantitative phytochemical screening, anticoccidial and antioxidant potential of eluted fractions was

evaluated by different assays. A total of 18 fractions from *F. racemosa*, 23 fractions from *C. fistula*, and 19 fractions from *S. cumini* were eluted from the crude extracts.

Among the *F. racemosa* fractions, fraction 19 at a dose of 30 mg/ml against *E. tenella* showed the greatest oocyst sporulation inhibition (67.11±2.18%). Fraction 7 showed the lowest effectiveness (3.31±2.37%) against *E. mitis* at a dosage of 2.5mg/ml. F-22 of *C. fistula* had the highest sporulation inhibition efficacy (69.11±2.18) and (69.56±1.14) % against *E. tenella* and *E. necatrix* respectively. F-4 showed the lowest effectiveness (4.562.05%) against *E. mitis* at a dosage of 2.5 mg/ml. F-15 of *F. racemosa* had the greatest DPPH radical scavenging inhibition with the lowest IC_{50} value (10.571.08) g/ml. This IC_{50} value's range (6.47–2.78) g/ml is quite close to that of standard ascorbic acid. F-15 of *F. raccemosa* has the highest total antioxidant capacity (45.41±1.55) *gAAE/mg*. Because their TAC is near to the antioxidant power of standard ascorbic acid (45.32±3.01). These fractions are considered to be powerful antioxidants. *S. cumini* F-19 (3.72.5) *gAAE/mg* was found to have the maximum ferric reducing capacity at a concentration of 600g/ml. The highest levels of the tested phytochemicals are present in various fractions of all selected plants. The highest total phenolic (120.00±2.13) and flavonoid (125.57±1.56) contents among all the eluted fractions of the selected plants were found in *C. fistula* F-22. It is suggested that particular plant methanolic extract bioactive fractions have the highest anticoccidial and antioxidant activity because they include medicinally significant phytochemicals.

Selected plants methanolic extract and their fractions different concentrations in comparison with anticoccidial drug amprolium were evaluvated for their anticoccicidial potential in broiler chicks (n=315). The experimental birds were divided into 21 groups labelled A-U on day 14. There were five chicks in triplicate in each group. With the exception of negative control group T, all experimental groups chicks received an oral infection from 7000 to 10,000 sporulated oocysts of a mixed *Eimeria* species. All groups, with the exception of negative control group T and medicated group S, were given with various supplements of plant extracts and their fractions orally at a dose of 1 ml/chick daily for 5 days beginning on day 4 post inoculation (PI), as the first evidence of infection occurred. Amprolium (1.25mg/ml) was administered to group S, the medication group. The efficiency of experimental dosages was assessed for 36 days on the basis of oocyst shedding, mean weight gain, diarrhea, feed consumption, feed conversion ratio, mortality, blood chemistry, histology etc. The acute toxicity of

extracts was examined in one-day-old broiler chicks (n=50). Total of ten groups, each containing 5 chicks.

All groups, with the exception of the control group, received a maximum oral dosage of 2,000 mg kg-1 b.wt of different extracts of selected plants and their anticoccidial fractions. For 14^{th} days, all the chicks were monitored for any clinical symptoms of toxicity. The anticoccidial index (ACI), anticoccidial sensitivity test (AST) and the percentage of optimal anticoccidial activity (POAA) was used to measure the drug resistance of *Eimeria* species. The maximum weight gain was observed in group N (447±4.04) and group M (444±3.05) treated with 20% and 10% of F-22 of C. fistula, respectively, on the fifth week. This weight gain was in similar range to that of group treated with amprolium (443±3.07) gm and the uninfected non-medicated control group (456±2.51) gm. Maximum mean feed consumption was observed in groups N (761±0.57) and M (759±0.57) treated with 20% and 10% of F-22 of *C. fistula*, respectively, at the fifth week of age. This was higher than the mean feed consumption in groups treated with amprolium (725±1.52)gm and within the range of the uninfected non-medicated control group S(762±1.15)gm. Group N, FCR results were the best. Among all experimental groups, group M treated with 10% of F-22 of *C. fistula* showed the greatest decrease in oocyst shedding. In the groups who received the best treatment, there was no death or diarrhea. Different concentrations of anticoccidial fractions from chosen plants were administered to groups, and normal histology, serum biochemistry, and hematological were observed in these groups.

Chemical characterization of the most active fractions was done by using UV-Vis, FTIR, and GC-MS analyses. The anticoccidial fractions UV-vis spectroscopy examination was carried out between 200 and 800 nm, and several absorption peaks were noted at various wavelengths. Based on typical peak values, the FTIR analysis was carried out to identify various functional groups. The spectrum was recorded by an FTIR spectrometer with a wavelength scan range of 400 to 4000cm^{-1}. Each fraction identified compounds were docked with target S-Adenosylmethionine synthetase by in silico molecular docking. Each fraction possesses amino acids, alkanes, alkynes, amines, aldehydes, carboxylic acid, and other compounds confirmed by FTIR analysis. Various common compounds, such as oleic acid, n-hexadecanoic acid, octadecanoic acid, trichloromethane, and etc were discovered by GC-MS analysis. Cyclododecane methanol of F-19 and oleic acid of F-15 of *F. racemosa* exhibit the greatest binding

affinities for the target S-Adenosylmethionine synthase among all docked compounds. These substances are perfect for the synthesis of plants based anticoccidial drugs.

Chapter 1

General Introduction and Objectives

GENERAL INTRODUCTION

Poultry industry

The poultry business plays a significant role in the agricultural economy and is a significant source of food for the entire world. By lowering poverty, the products of this business contribute to the economic development of emerging nations (Gueye, 2005). Poultry includes all native birds that are raised for their meat and eggs, such as chickens, turkeys, ostriches, ducks, and others. Chickens are the most significant source of animal proteins world wide (Bogosavljevic-Boskovic *et al*., 2010). Poultry production in Pakistan accounts for 2% of the country's GDP (Yousaf *et al*., 2017). Numerous illnesses, particularly the most common and harmful one, coccidiosis, have a negative impact on commercial chicken outputs globally both in the past and in the present (Abdisa *et al*., 2019 and Soutter *et al*., 2020).

Coccidiosis

Parasitic disease coccidiosis is one of the illnesses with the most financial effects (Mohammed and Sunday., 2015; Olanrewaju and Agbor., 2014). This fatal disease is caused by an intracellular protozoan parasite of the genus *Eimeria* (Taylor *et al*., 2007). Over 1000 different species of *Eimeria* parasitize the intestinal epithelial cells of various vertebrate species like dogs, cats, horses, rabbits, cattle, sheep, pigs, turkeys and chicks (Witcombe and Smith., 2014). Seven species of *Eimeria* like *E. acervulina*, *E. praecox*, *E. tenella*, *E. necatrix*, *E. mitis*, *E. maxima* and *E. brunetti* infect chicks (Abdisa *et al*., 2019). These species of *Eimeria* differ from one another in terms of fertility, pathogenicity, and infection sites (Chapman, 2014). *E. tenella* is the most prevalent and dangerous species among these, leading to significant mortality, morbidity, and hemorrhagic lesions (Gyorke *et al*., 2016). The least pathogenic are *E. praecox* and *E. hagani*, while the mild pathogenic are *E. acervuline*, *E. mitis*, and *E. mivati* (Jadhav *et al*., 2011). All *Eimeria* species have strong cell walls and are round. Their oocysts are shaped like an oval. *E. maxima* is the biggest species (30.5 x 20.7 m), and *E. mivati* is the smallest (15.6 x 13.4 m) (Clark and Blake, 2012). The host, infection locations in the gut, kind of lesion, sporulation period, schizont size, and other

characteristics of the different species of *Eimeria* vary from one another (Abdisa *et al*., 2019).

Life cycle

Eimeria goes through two developmental stages during its 4-w6-day life cycle, known as the exogenous and endogenous stages (Blake and Tomley., 2014). During the exogenous phase of the *Eimeria* life cycle, the unsporulated oocysts from the chicks are excreted in the faeces, and in the external environment, the oocysts undergo sporulation in an aerated moist environment. Each of the four sporocysts, which are found in sporulated oocysts, contains two sporozoites. The endogenous phase of the cycle initiated after the entrance of sporulated oocysts in the GIT of host. The microenvironment of host tract leads to the excystation of sporulated oocysts, which releases sporozoites that attack and kill the intestinal epithelial cells. After several cycles of asexual reproduction, sexual reproduction occurs *i.e*., gametogony and fertilization. The shedding of unsporulated oocysts occur through faeces (Lal *et al*., 2009, McDougald., 2013, Gilbert *et al*., 2011).

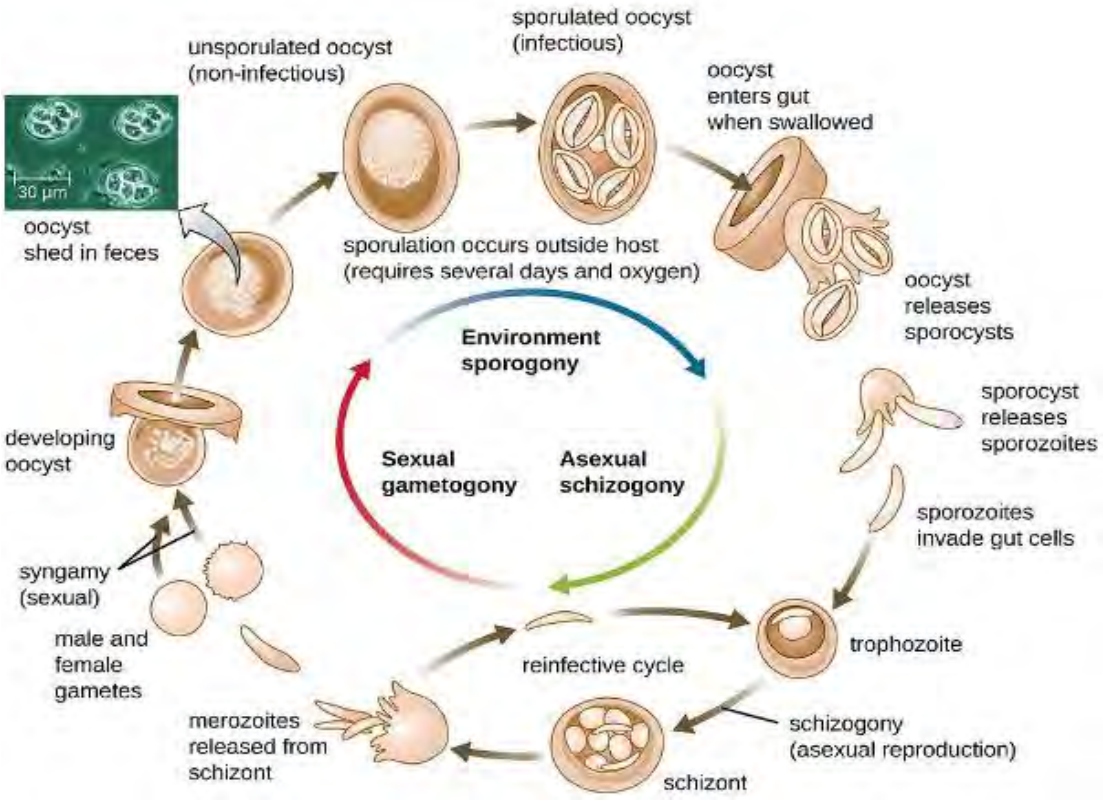


Figure 1.1: *Eimeria* species life cycle (https://www.dsm.com/anh/challenges/supporting-animal-health/coccidiosis-in-chickens.html)

Pathogenicity

The exact process by which *Eimeria* species causing disease is unknown, although sporozoites of these species form holes or lyse the cell membranes in the epithelial lining of the host intestine by means of a protein called a membrane attack complex (MACPF) that is included in perforin (Sassmannshausen *et al*., 2020). A calcium-regulated protein is secreted by the micronemes and rhoptries of *Eimeria* species to aid in host cell invasion, parasite movement, and egress (Dubois and Soldati-Favre, 2019). Adhesion molecules, such as proteins AMA1, bind directly to the adhesion site during the first stage of invasion (Tyler *et al*., 2011). The majority of *Eimeria* species infect chicks between the ages of 3 and 18 weeks. (Morris & Gasser, 2006). Necrotic enteritis, bloody diarrhea, lack of appetite, reduced feed intake, dehydration, bending, weight loss, rough feathers, depression, exhaustion, and death follow a huge invasion of the sporulated oocysts and sporozites (Conway and Mckenzie, 2007, Taylor *et al*., 2007).

Transmission

Poultry coccidiosis is spread from one chick to another by ingesting sporulated oocysts through feco-oral route, direct and indirect contact with excretions of infected chicks, as well as by people moving between farms. Consuming food and water contaminated with sporulated oocysts and using infected utensils are methods of spreading disease among chicks (Chapman, 2002; Sharman *et al*., 2010). Oocysts were distributed in farm buildings via mechanical ventilation system pests (Taylor *et al*.,2007). Beetles, flies, cockroaches, rodents, pets, and other mechanical vectors play a part in the spread of *Eimeria* oocysts (Charlton, 2006). The oocysts' resistance to extreme environmental conditions and disinfectants is a significant component in the transmission of the parasite coccidia. Broiler litters frequently contain the *Alphitobius diaperinus* beetle, which transmits *Eimeria* oocysts (Wiley and Sons., 2020).

Geographical distribution

Growing chicks throughout the world frequently contract coccidiosis (Conway and Mckenzie, 2007). Tropical and subtropical locales with ideal climatic conditions

for oocyst sporulation are home to an endemic form of poultry coccidiosis (Obasi *et al*., 2006).

Prevalence

The frequency of coccidiosis in broilers varies globally, with estimates ranging from less than 10% to more than 90% (Bachaya *et al*., 2015). *Eimeria* species are more common in young chicks than adults in Pakistan (Jamil *et al*., 2016). High levels of precipitation and humidity accelerate the spread of this illness (Sohail *et al*., 2019). Because of this, the prevalence rates of certain *Eimeria* species vary significantly across different geographic regions (Zhang *et al*., 2013).

Economic importance

Coccidiosis led to significant financial losses globally, with an estimated cost of more than 3 billion USD each year (Mokhtar *et al*., 2016; Dkhil *et al*., 2012). Coccidiosis, for example, may cost more than half of a yearly profit in Central Java (Pawestri *et al*.,2020). Poultry coccidiosis is a significant parasitic illness with economic significance in Pakistan (Ullah *et al*., 2014). Due to the decline in output brought on by high mortality, low weight increase, and high feed conversion rate (FCR), the commercial broiler sector might suffer economic losses of up to 95.6%–98.1% (Bera *et al*., 2010). The expense of immunization, decontamination, restocking, eradication, lost income and etc are only a few of the financially detrimental effects of this illness on the poultry industry (Thrusfield, 2005).

Diagnosis

Clinical symptoms such the presence of blood in stools and diarrhea, coprology, path morphological and path histological techniques, as well as several biochemical and molecular procedures, are the major diagnostic approaches for coccidiosis in poultry (Conway and Mckenzie, 2007). Additionally, the most popular technique for diagnosis is serology (Wakenell, 2010). Additionally, the flotation technique for oocyst detection in faeces is used (Conway and Mckenzie, 2007). Identification of *Eimeria* species can be aided by observing the size, shape, and minimum sporulation time of the oocyst (Arabkhazaeli *et al*., 2011). Through postmortem observation, lesions, their location, appearance, severity, and other related changes in birds may be examined

(Conway and Mckenzie, 2007). When examining a lesion under a microscope, the number of parasite stages such as oocysts, schizonts, or gametocytes is counted (Mcdougald, 1998). Various molecular techniques like PCR, multiplex PCR, RT PCR and etc are used for the diagnosis of coccidiosis (Carvalho *et al*., 2011; Vrba *et al*., 2010; Barkway *et al*., 2015). Molecular techniques are advantage over traditional ones, because they identify the genetic sequence of the *Eimeria* species (Ogedengbe *et al*., 2011). Coccidiosis can be diagnosed by histopathological analysis of several tissues (Olabode *et al*., 2020).

Different anticoccidial medications of the ionophores category have been used in the past and today, including amprolium, monensin, lasalocid, narasin, maduramicin, nicarbasin, and robenidine (Kahn, 2008). The ion gradient across the cell membrane is disrupted by ionophores, which also cause the parasite to burst (Fanatico, 2006). Synthetic medications such as sulfonamides, amprolium, and others disrupt the parasite's mitochondrial respiration, folic acid pathway, and thiamine absorption as another class of anticoccidial substances (Allen and Fetterer, 2002). Today's significant issue is brought about by the development of drug-resistant strains of the coccidia parasite and by the ongoing usage of anticoccidial medications (Roy, 2007). *Eimeria* species acquired resistance in Pakistan as a result of long-term, regular usage of salinomycin to treat coccidiosis (Abbas *et al*., 2011; Gyroke *et al*., 2013). Other than drug resistance, anticoccidial medications have no detrimental effects on the health of chicks (Hafeez *et al*. 2020). Because of this, the European Union (EU) outlawed the use of antibiotics as animal feed additives (Nasir *et al*., 2020).

Vaccination and immunization

Researchers also developed early vaccinations against this illness in addition to developing treatments and resistance in *Eimeria* species (Yim *et al*., 2011). To combat avian coccidiosis, many immunization techniques have been developed. Livacox and Paracox vaccinations are also in practiced (Shirley and Harvey, 2000; Arabkhazaeli *et al*., 2014). Chicks are immunized against coccidiosis using two different types of vaccines: attenuated and virulent (Chapman *et al*., 2002). These are attenuated, pathogenically inactive and strains with a poor capacity for reproduction. However, production costs for attenuated vaccines are much greater due to their limited ability to

reproduce. Viable vaccinations contain anticoccidial sensitive strains. The potential of anticoccidial sensitivity strain vaccinations to alter the level of resistance in certain coccidial populations is their primary benefit (Mathis and Broussard, 2006).

Prevention and control

Due to *Eimeria* species resistance development, many studies on experimental infections and anticoccidial medication trials have been done in Pakistan (Zaman *et al.*, 2012). It is far simpler to avoid poultry coccidiosis than to treat it. Because the majority of the harm is already done by the time coccidiosis symptoms appear. Nowadays, nearly all broiler flocks are frequently treated with preventative drugs (McDougald, 1987). Coccidiosis can be prevented with the use of good management methods (Ashenafi *et al*., 2004). Litter should always be kept dry in chicken farms, and extra care should be taken with litter near water and feeding ditches (Taylor *et al*., 2007). The key management practices for the disease's prevention is poultry farms should have adequate air, dry, clean litter, and particular care during the rainy season, isolation of clinically infected birds, proper stocking density in poultry farms, washing and cleaning of feeders and drinkers (Ashenafi *et al*., 2004; Roy and Chauhan, 2007).

Natural feed additives

Coccidiosis has been treated using plants, herbs, and ethnobotanicals ever since the dawn of humans. Natural goods, or those derived from plants, play a significant role in the creation of commercial pharmaceuticals. Several antioxidant-rich plants may be helpful in treating chicken coccidiosis (Allen and Fetterer, 2002). By giving oregano extract to hens with coccidiosis, researchers were able to combat *E. tenella* by observing increase in body weight and feed conversion ratios (Abdullah *et al*., 2009). Chicks treated with *Moringa stenoptela* leaf powder dramatically decreased the cecal lesion scores and oocysts no of *E. tenella* in faeces similar to those treated with amprolium (Adamu *et al*., 2013).

Herbal drugs

Apacox (GreenVet, 2016) a formulation of BP made from *Biden pilosa* and other plants (Yang *et al*., 2015), a combination of *Rhus chinensis*, *Terminalia chebula*, and *Quercus infectoria* (Kemin Industries, USA) (Gokila *et al*., 2014) and cocci-Guard

(DPI Global, USA) are the four anticoccidials with plant origins that are currently in use. Prior to future uses, certain problems related to the use of plant-derived natural products against coccidiosis, such as active compound discovery, mechanism of action, anticoccidial efficacy, and compound and plant extract cost effectiveness, must be solved. About 68 plants and their phyto compounds have been systematically evaluated against *Eimeria* species up to this point (Abbas *et al*., 2012; Bozkurt *et al*., 2013). The plants *T. vulgaris*, *D. febrifuga*, *C. sinensis*, *A. officinalis*, wheat bran, and others that were evaluated specifically target the sporozoites stage of the parasite (Thangarasu *et al*., 2016).

Oxidative stress caused by *Eimeria* parasite

Vertebrate are protected by the innate immune system via the oxidative burst-induced production of reactive oxygen species. Extremely reactive oxygen species harm the pathogen's proteins, lipids, and DNA. In the absence of antioxidant defenses, reactive oxygen species produce oxidative stress and damage to host tissues (Monaghan *et al*., 2009; Costantini *et al*., 2010).

Plants as a source of antioxidants

Natural or synthetic antioxidant bioactive substances prevent and postpone the oxidation of molecules (Halliwell *et al*., 1995). BHT (Butylated Hydroxytoluene), Propyl gallate, BHA (Butylated hydroxyanisole) and other synthetic antioxidants are often utilized. In addition to their benefits, they might have negative side effects include liver damage and other health issues. Thus, the creation of safer antioxidants has expanded due to plants. Tamarind, cardamom, lemon grass, and other naturally antioxidant-rich plants are commonly used as traditional medicines (Javanmardi *et al*., 2003).

Cluster fig (*Ficus racemosa*), a member of the Moraceae family, was one of the plants chosen for the current investigation (Irshad *et al*., 2012). This plant, a large deciduous tree, is found throughout the Himalayan region in India (Kumar *et al*., 2006). This grows in damp environments like stream banks, evergreen and deciduous woods, and deciduous forests. planted often in several places for its shadows and delicious fruits (Bahorun *et al*., 2005; Siddhuraju *et al*., 2002). Various cluster fig components,

including as the barks, stems, and roots, have been employed in the past to separate distinct bioactive chemical substances. Tetra triterpene, glaunanol acetate, and racmosic acid are all components of the *F*. racemosa leaf extract. Hentriacontane, -sitosterol, tiglic acid, and other significant chemicals were found in the fruit of *F. racemosa* (Joseph and Raj, 2010).

F. racemosa is a traditional medication used in the subcontinent to treat a variety of illnesses. Similar to their bark, which contains potent analgesic, antihelminthic, anticholinesterase, antidiuretic, and memory-improving properties. Additionally, the bark extract aids in the treatment of piles, urological diseases, diabetes, and diarrhea. *F. racemosa* roots are frequently used to treat piles, diabetes, diarrhea, and inflammatory glandular enlargements. Diabetes, dysentery, and different inflammatory glandular enlargements can all be treated with their roots. Treatments for vaginal problems, traumatic swelling, anti-inflammatory, antibacterial, hepato protective, anti-hyperglycemic, and hemorrhoids frequently involve the use of cluster pig latex and leaves. This plant's fruits are commonly used to treat renal and spleen diseases (Paarakh, 2009; Deep *et al*., 2013).

Another plant chosen for the study is *Syzygium cumini*, commonly known as jamun of the Myrtaceae family. This is a large, evergreen shrub having pharmacological qualities that are widely recognized. Often found in Afghanistan, India, Thailand, Philippines, and other places. Because jamun contains tannins, oxalic acid, gallic acid, and malic acid, it has significant therapeutic value (Quiroz-Castaneda and Dantan-Gonzalez 2015). The pharmacological properties of tannin, flavonoids, betulic acid, and essential oils include anti ulcerogenic, gastro protective (Kirtikar *et al*., 1975; Satyavati and sharma 1989), anti-malarial (Gokila *et al*., 2014), antibacterial (Siddhuraju *et al*., 2002) and anti-infective (DPI, 2016). All parts of the *S. cumini* plant, notably the seed, are used to treat diabetes mellitus. Strong anti-inflammatory, anti-fungal, anti-leishmanial, anti-HIV, antifertility, anorexigenic, gastroprotective, anti-ulcerogenic, and radio protective properties are all treated by the *S. cumini* plant. This plant also contains anti-diarrheal, free radical, and nitric oxide scavenging qualities (Irshad *et al*., 2012).

The third plant chosen for this study is *Cassia fistula*, often known as golden shower of the Fabaceae family. This plant is frequently found in places like Asia, South Africa, China, and Brazil. This is a tree of average height with medicinal properties in all of its parts, including cooling, purgative, tonic, anthelminthic, antiperiodic, diuretic, anti-inflammatory, antimicrobial, anticancer, and antioxidant (Kirtikar *et al*., 1975; Satyavati and Sharma 1989; Siddhuraju *et al*., 2002; Irshad *et al*., 2012; Irshad *et al*., 2013). It also causes the contraction of skin cells and other body tissues. The leaves of the *Cassia fistula* are effective in treating fever, cough, burning, and skin conditions. Their blooms are used to cure heart ailments. Anthraquninone and tannin derivatives from the *C. fistula* plant are significant pharmaceutical components (Siddhuraju *et al*., 2002). According to our knowledge, this is the first research to compare the crude extracts and fractions of the selected plants with the medicine amprolium, which has never been used to treat coccidiosis.

Aims and Objectives

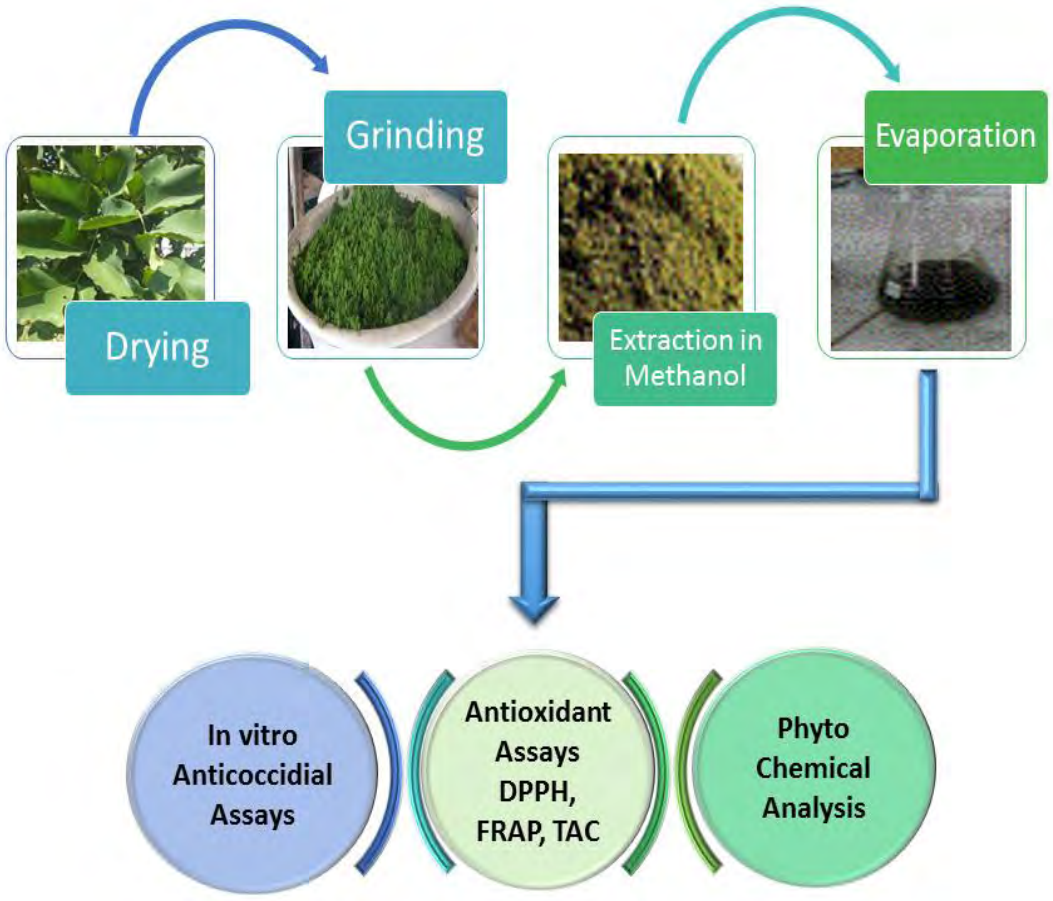
The following are the primary aims and objectives of the current study;

- Initial screening and research for phytochemicals of aqueous and methanolic crude extracts of *Ficus racemosa*, *Cassia fistula* and *Syzygium cumini* leaves.
- Fractionation of selected plants crude methanolic extracts and identification of anticoccidial and antioxidant fractions.
- *In vivo* evaluation of the anticoccidial fractions along with their crude extracts in comparison with coccidiostat amprolium.
- Chemical characterization of the active fractions and in silico molecular docking of identified compounds with target S-Adenosyl methionine synthetase.

Chapter 2

Title: *In vitro* anticoccidial, antioxidant activities and biochemical screening of methanolic and aqueous leaves extracts of selected plants

Graphical Abstract



ABSTRACT

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

INTRODUCTION

A number of pathogens threaten poultry health and welfare. Among all these, the most important is a protozoan parasite of the genus *Eimeria*. This parasite invades and replicates within the epithelial cells of the gut, needing costly treatment and reducing the productivity of both layers and broilers. The loss of this infection and the cost of different interventions causes more than 3 billion dollars of global loss annually (Dalloul *et al*., 2006). In recent years, many poultry farms have been subjected to maximum outbreaks of coccidiosis. This devastating, deadly, and economically damaging disease infects birds all over the world. Coccidiosis causes bloody diarrhea, weight loss, reduced feed conversion ratio, poor growth, high morbidity, and mortality. In this disease, maximum spread of infection occurs by the spread of infectious oocysts to the surrounding environment (Kadykalo *et al*., 2018). Coccidiosis is an avian parasitic disease caused by 13 different species of *Eimeria*. In Pakistan, approximately 0.96-1.08 million tonnes of meat were produced by this sector in 2014-2015. Now days due to advancements in biotechnology, commercial poultry farms are being reared into conventional poultry farms (Abbas *et al*., 2012). Priced anti coccidial drugs and high mortality rate cause 240 million dollars' loss in Java and US$ 0.005 in Pakistan (Pawestri *et al*., 2020; Jan *et al*., 2013). The extensive use of anticoccidial drugs such as Amprolium, Clazuril, Diclazuril, Monensin, Salinomycin, Spiramycin, Sulfadimethoxine, and Toltrazuril leads to drug resistance in chicks. In addition, the residues of coccidiostats and antimicrobial drugs in poultry products may pose a public health concern (Mund *et al*., 2017).

The currently available drugs and vaccinations for control of coccidiosis are costly, seriously risky and parasites producing resistance, therefore there is a need for exploring new agents such as use of herbal drugs against *Eimeria* species (Al-Quraishy *et al*., 2020; Abbas *et al*., 2019). Due to the development of drug resistance in Eimeria species, many researchers are in search of alternative strategies to combat coccidiosis. Among all such strategies, the best one is the identification of anticoccidial natural compounds (Pop *et al*., 2019). In many developing countries, medicinal plant use has been widely proposed. Nowadays, an increasing trend for the utilization of medicinal plants in the industrial sector has been seen in the different sectors, such as extraction and development of several drugs having disease curing as well as biological properties (Sharifi-Rad *et al*., 2018). Most illnesses, including coccidiosis, are primarily

associated with oxidative stress induced by free radicals. Different antioxidant compounds are present in plants which have anticoccidial and therapeutic effects (Awaad *et al*., 2016). In the majority of pathological diseases, pathophysiology is mainly due to oxidative stress (Chang *et al*., 2002). Plants have a diverse group of potent antioxidant compounds like terpenoids, phenolics, vitamins, and nitrogen compounds (Cai *et al*., 2003). Natural antioxidants possess a variety of biochemical properties, like scavenging of free radicals, inhibition of the production of reactive oxygen species, and amendment of cellular redox potential. Inflammation stimulated in response to an infection has been associated with various diseases such as diabetes, cancer, atherosclerosis, and hypertension (Schmid, 2006; Ahmed *et al*., 2018). Various plants with anti-inflammatory effects are used as alternatives for the treatment of inflammation, and thousands of reports exist on the anti-inflammatory properties of various plant tissue extracts (Ramírez *et al*., 2012). In the current study, the crude methanolic and aqueous leaf extracts of three plants, *i.e*., *Ficus racemosa, Cassia fistula* and *Syzygium cumini* were evaluated for their antioxidant capacity against coccidiosis. The *F. racemosa* (Moraceae) commonly known as cluster fig. *F. racemosa* is an important medicinal plant, found in India, Australia, and Southeast Asia. Many active constituents that have been isolated from various parts of this plant possess useful pharmacological activities. This is a well-known medicinal plant rich in phenolic and flavonoids that exhibit strong antioxidant and antimicrobial activities, thrombolytic, membrane stabilizing, antioxidant, antimicrobial, analgesic, and hypoglycemic activity (Mohiuddin *et al*., 2020, Bagyalakshmi *et al*., 2019).

Cassia fistula (Fabaceae family), commonly known as amaltas. Cultivated in southern Pakistan to Sri Lanka and eastward to India (Gupta, 2010) possess medicinally important phytochemicals (Kamath *et al*., 2019). *C. fistula* is used for the treatment of inflammation, chlorosis, constipation, colic, and urinary disorders due to the presence of bioactive polyphenols (Haider *et al*., 2018). *S. cumini* (Myrtaceae) is widely distributed in tropical and subtropical areas, commonly known as Jamun, and has a wide range of therapeutic characteristics (Eshwarappa *et al*., 2014). The extracts of various parts of *S. cumini* contain phytochemicals including tannins, anthocyanins, terpenes, flavanols, and aliphatic-acids. All parts of S. cumini are rich in polyphenols (Baliga *et al*., 2011). This work was aimed at investigating anticoccidial, antioxidant activities and biochemical screening of crude methanolic and aqueous leaf extracts of

selected plants since no previous studies have been recorded on their anticoccidial effects. The present study will have great significance in evaluating these plants as a potential source of drug formulation for coccidiosis.

MATERIALS AND METHODS

Plants collection, identification and extraction

During June to September 2018, the fresh leaves of selected plants *Ficus racemosa, Cassia fistula* and *Syzigium cumini* were collected from different localities of Quaid-i-Azam university (QAU), Islamabad, Pakistan in sterilized bags (Figure 2.1). After collection leaves were cleaned with tap water then rinsed with distilled water. The samples were shade dried and then powdered by electric blender and stored at room temperature in air tight container until it is used for extraction (Kavitha *et al*., 2020).

(a) **(b)** **(c)**

Figure 2.1: Selected plants fresh leaves **(a)** *Cassia fistula* **(b)** *Ficus racemosa* **(c)** *Syzigium cumini*

Plants were identified through flora of Pakistan and taxonomist Dr. Mushtaq Ahmad of plants sciences department Quaid-i-Azam university Islamabad, Pakistan (Abdullah *et al*., 2018). Plants specimen were deposited in herbarium of Pakistan under voucher code of 130863 for *F. racemosa,* 130862 for *C. fistula* and 130861 for *S. cumini*.

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). In maceration extraction technique 150gm of leaves powders were taken from three selected plants in separate conical flasks and permitted to soak in 500ml of methanol and water separately. Contents were kept for 2 days in conical flasks and shaken vigorously. The mixture was stirred and then filter under control temperature (45°C) using Whatman No 1 filter paper. Solvents were fully evaporated using a rotary evaporator at 50rpm and 40°C. The methanolic and aqueous extracts produced were

kept in glass beakers covered with aluminum foil in refrigerator at 4°C for further processing (Sultana *et al*., 2013; Ezeh *et al*., 2020). Extract yield was calculated in grams by using following formula and expressed as percentage (Gahlot *et al*., 2018; Egua *et al*., 2020).

Percentage yield=Practical yield/actual weight of plant engaged in extraction× 100

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). For the determination of *In vitro* anticoccidial activities of selected plants two assays were performed *i.e*., oocysts sporulation inhibition and sporozites viability inhibition assays.

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 oocysts with 4 sporocysts was considered sporulated. Oocysts with damaged wall were not considered in counting (Abbas *et al*., 2020). 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 (1.25*mg/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 (1.25*mg/ml*) as a positive control were used. The complete set up was examined for the viability of sporozoites after 48 hours. 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).

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

Figure 2.2: *In vitro* anticoccidial assays of selected plants crude extract different concentrations.

Determination of antioxidant potential of crude extracts

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.

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

Where *A0* is the absorbance of the control and *A1*is the absorbance of the sample extracts. Each sample antioxidant activity was expressed in terms of IC_{50}. IC_{50} values expressed as $\mu g\ AAE/mg$ of extracts. 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). Different concentrations *i.e*., (100, 300 and 600$\mu g/ml$) of extracts and ascorbic acid was prepared in distilled water (1ml). 2.5ml of phosphate buffer (0.2M, pH 6.6) and potassium feericyanide (1%) solution was mixed with extracts. The mixture was then placed in incubator for 20 minutes at 50°C. After incubation 10% of tri chloro acetic acid (2.5ml) was added. Mixture was centrifuged for 10 minutes at 3000rpm. Supernatant was collected, mixed with distilled water (2.5ml) and 0.1%(w/v) ferric chloride solutions (0.5ml). Absorbance was measured by spectrophotometer at 700nm.

Total antioxidant capacity estimation

The total antioxidant capacity of extracts was evaluated by phospho molybdenum method (Sakat *et al*., 2010). In this method reduction of Mo (VI) to Mo (V) by the formation of a green colored phosphate/Mo (V) complex (Jafri *et al*., 2014). 180µl of phospho molybdenum reagent is mixed with 20µl of test samples (4mg/ml). Then incubated at 95ºC for 90 minutes in water bath. Samples were cooled at room temperature after incubation and transferred to 96 well plates. Ascorbic acid and DMSO were taken as positive and negative controls, respectively. By using microplate reader (Platos R 496) absorbance was measure at 630nm. Results have been expressed as ascorbic acid $\mu g/ml$ of extract. The experiment was conducted in triplicate.

Phytochemicals screening of crude extracts

For the preparation of stock solution of concentration 1%(v/v), one gram of methanolic and aqueous crude extracts of selected plants leaves was dissolved in 100ml of its own mother s' solvents. The obtained stock solution of crude extracts was subjected to preliminary phytochemical screening (Hossain and Nagooru., 2011).

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 using standard methods (Sofowra, 1993; Joshua *et al*., 2020).

Test for Phenols and Tannins

Ferric chloride test

Methanolic and aqueous crude extracts was mixed with 2% solution (2ml) of $FeCl_3$. The presence of phenols and tannins was stated by a blue-green or black color.

Test for flavonoids

Alkaline reagent test

Crude extracts were mixed with 2ml solution of 2% NaoH. An intense yellow color was produced that turned colorless on addition of few drops of diluted acid, indicating the existence of flavonoids.

Test for Saponins

Froth test

All extracts were mixed separately in test with 5ml of distilled water and srongly shaken. Stable foam formation was taken as an indication of saponins existence

Test for glycosides

Liebermann's test

Chloroform (2ml) and acetic acid (2ml) was mixed with all the crude extracts separately.

The mixture was icecooled. Added cautiously concentrated H_2SO_4. A shift in color fro m violet to blue indicates that glycosides are present.

Test for Terpenoids

Salkowski's test

Chloroform (2ml) was mixed with crude extracts. Then concentrated H_2SO_4 (2ml) was added and shaken vigorously. A reddish brown color stated the existence of the glycoside part of the steroidal ring.

Test for alkaloids

Mayer's test

To all the extracts 2ml of 1% HCL was added and heated. Then Mayer s' and Wagner reagents were added to the mixture. The resulting precipitate turbidity was taken as proof of alkaloid existence.

Test for proteins

Millon's test

White precipitate appeared when extracts was mixed with 2ml of Millon s' reagent. White precipitate turned red on heating confirming protein existence.

Test for Carbohydrates

Fehling's test

To the mixture of Fehling A and Fehling B reagents, 2ml of crude extract was added and boiled gently. The presence of reduced sugars was stated by a brick red precipitate at the bottom of the test tube.

Quantitative Analysis

Determination of total flavonoid contents (TFC)

The colorimetric technique of aluminum trichloride (AlCl3) was used to deter mine the totalflavonoids contents with slight changes according to system suitability (Chang et al., 2002; Kaneria et al., 2014). 20µl of the test sample (4 mg/mL) along with positive and negative controls i.e. DMSO and quercetin (1mg/ml) were taken in 96 well plate and incubated for 30 minutes at 37°C.

It was followed by adding 10µl of aluminum chloride (10%),10µl of potassium acetate (98.15g/l) and raising final volume with distilled water up to 200µl. Absorbance was measured by micro plateplate reader at 405nm and triplicate results were analyzed as µgQE /mg extracts (Chanda et al., 2012). Results were expressed as Mean±SD

Determination of total phenolic contents (TPC)

Folin-Ciocalteu reagent technique with slight modification was used to determine the total phenol content. 1mg of each crude extract was dissolved in 1ml of its mother solution. Then 10% Folin-Ciocalteu reagent was prepared by addition of 10ml Folin-Ciocalteu reagent with 90ml of water. Then, by dissolving Na_2CO_3 (3g) in water (50 mL), 5% Na_2CO_3 (3g) was prepared. From each crude sample 200µL was taken in separate test tubes and 10 percent Folin Ciocalteu reagent (1,5 mL) was added.

All the test tube was kept for 5 minutes in a dark place. After 5 minutes to all the test tubes 5% Na_2CO_3 (1.5 mL) was added and mixed well. Again all the test tubes were kept for 2 hours in the dark place. The absorbance was measured with a UV

spectrophotometer at a steady wavelength of 750nm. Results were expressed as Mean±SD (Jagadish *et al*., 2009).

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 plant, *i.e., F. racemosa*, *C. fistula* and *S. cumini* 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 2.1).

Table 2.1: Percentage yield of the selected plant extracts

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

.

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* (Figure 2.3 and 2.4).

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* (Figure 2. 5).

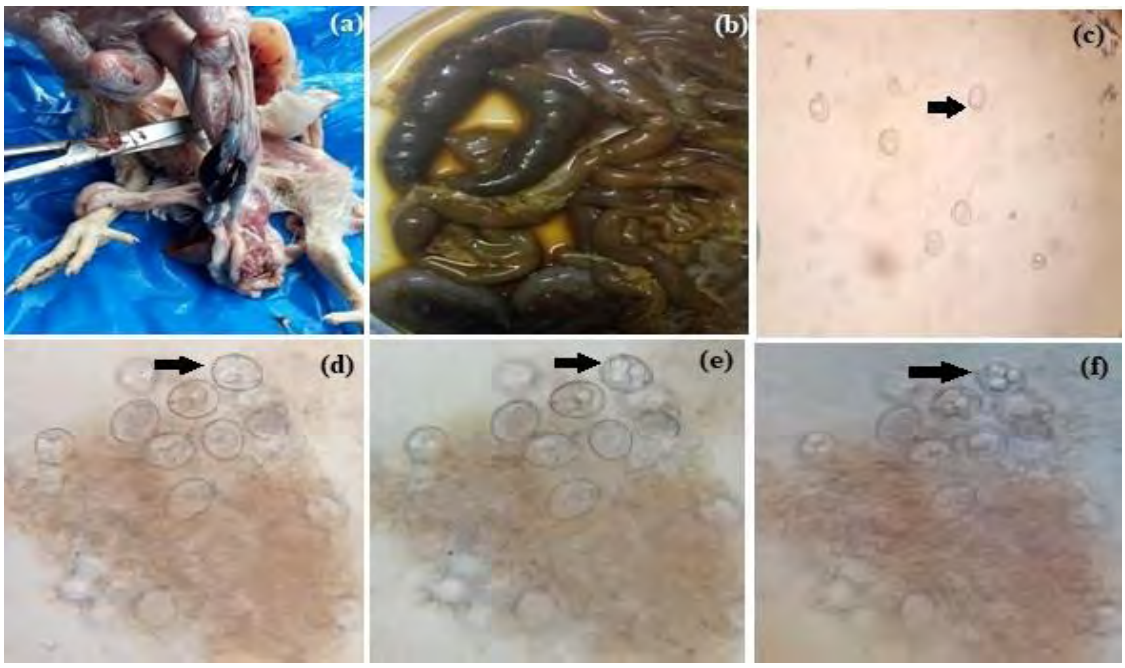


Figure 2.3: (a) Coccidiosis infected chick **(b)** Caecum and intestine of infected chick **(c)** un sporulated oocysts **(d), (e)** and **(f)** Sporulated oocysts

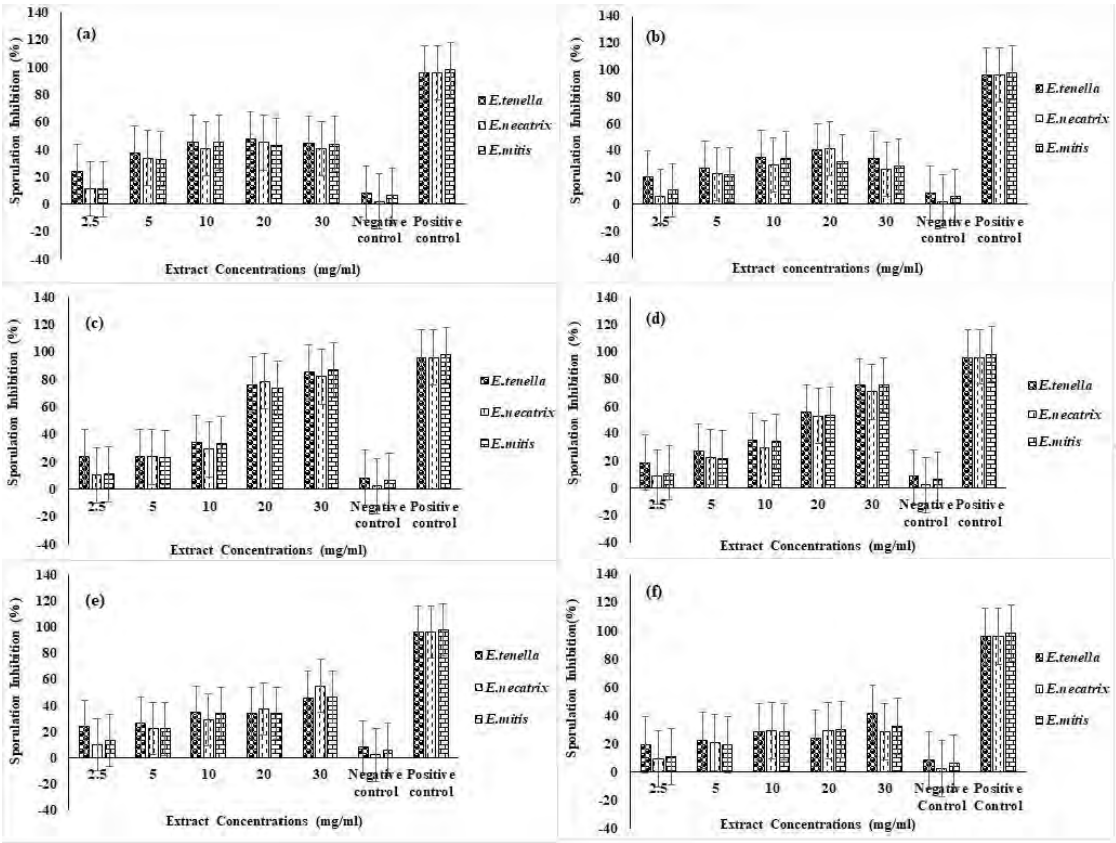


Figure 2.4: (a), (b): Effect of *F. racemosa* methanolic and aqueous leaves extracts

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.

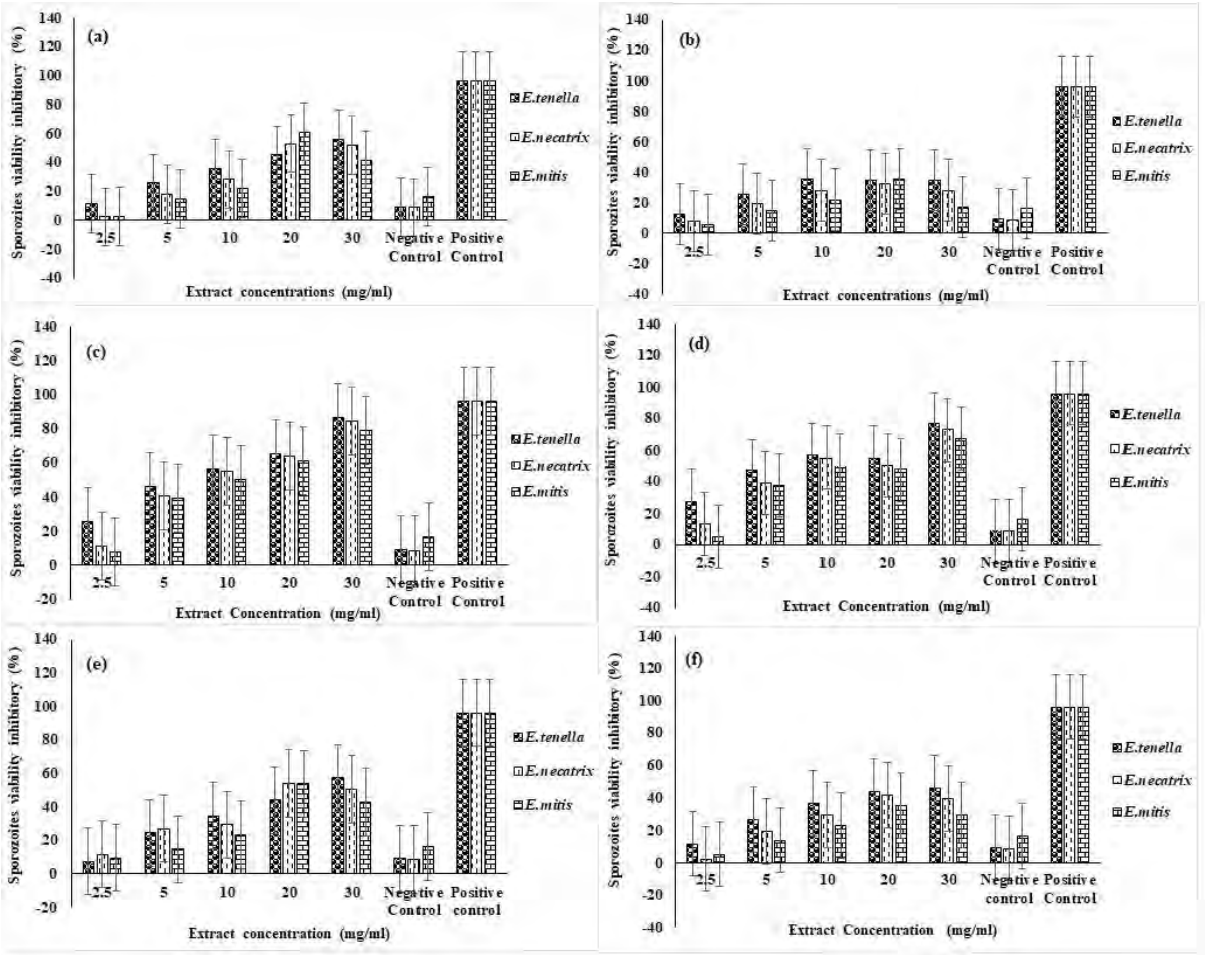


Figure 2.5: (a), (b): Effect of *F. racemosa* methanolic and aqueous leaves extracts different concentration on sporozoites viability inhibition of different *Eimeria* species. **(c), (d).** Effect of *C. fistula* methanolic and aqueous leaves extracts different concentration on sporozoites viability 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.

Antioxidant potentials of crude extracts

DPPH radical scavenging activity

The free radical scavenging activity of aqueous and methanolic leaves extracts of selected plants was evaluated by the discoloration of DPPH reagent. A color change from purple color to yellow was observed. The results revealed that highest radical scavenging activity was observed by *F. racemosa* with (67.82±0.00%) inhibition followed by *S. cumini* and *C. fistula* (Table 2.2). The half maximal inhibitory concentration (IC_{50}) values were calculated. The lowest IC_{50} values of methanolic extracts of *F. racemosa* and *C. fistula* exhibited the highest radical scavenging activity. While aqueous extracts of selected plants showed lowest inhibition at highest IC_{50} values (Figure 2.6).

Table 2.2: DPPH radical scavenging activity (%) of different extracts of selected plants.

Parameters		***F .racemosa***	***C. fistula***	***S. 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[1]	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]

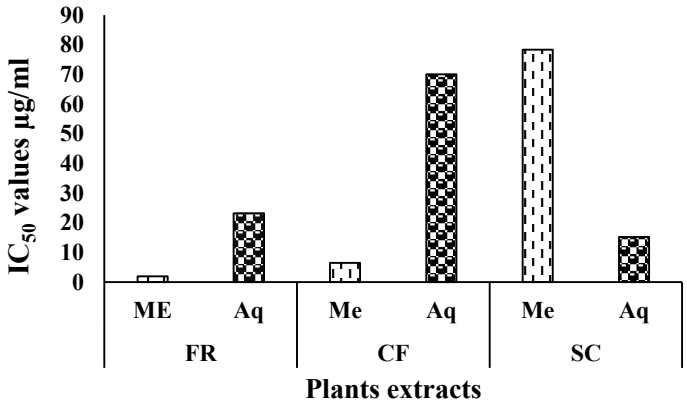


Figure 2.6: IC_{50} values for methanolic and aqueous extracts of selected plants

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.

Reducing power assay

To test the reducing ability of the extracts, reducing power assay is used. In this assay due to the reducing power of extracts, Fe^{3+} (potassium feericyanide) is converted into Fe^{2+} (potassium ferrocyanide), Fe^{2+} then reacts $Fecl_2$ (ferric chloride) and results the formation of complex ferrous. The results of the present study revealed that plant extract exhibit increase in reducing power as the concentration of extract increased. The highest reducing power was of the methanolic leaves extract of *C. fistula* (2.17±0.01) at 600*µg/ml* concentration, statistically similar to the reducing power of ascorbic acid (2.88±0.06) at the same concentration (Table 2.3).

Table 2.3: Total reducing power of different solvents extracts of selected plants

Conc. *µg/ml*	**Solvents**	**Absorbance of extracts and standard (Mean ± SD)**			
		FR (µg/ml)	**CF (µg/ml)**	**SC (µg/ml)**	**Ascorbic Acid**
100	Me	0.07 ± 0.00^{ij}	0.81 ± 0.00^{f}	0.07 ± 0.00^{ij}	0.92±0.01
	AQ	0.02 ± 0.01^{j}	0.13 ± 0.06^{hi}	0.07 ± 0.00^{e}	
300	Me	0.23 ± 0.02^{h}	1.22 ± 0.02^{d}	0.23 ± 0.02^{h}	1.36±0.03
	AQ	0.04 ± 0.01^{ij}	1.26 ± 0.09^{d}	0.23 ± 0.02^{c}	
600	Me	0.53 ± 0.02^{g}	2.17 ± 0.01^{b}	0.53 ± 0.02^{g}	2.88±0.06
	AQ	0.05 ± 0.02^{ij}	2.16 ± 0.02^{b}	0.53 ± 0.02^{a}	

Same superscript letter values are not significantly different at $P\geq0.05$.

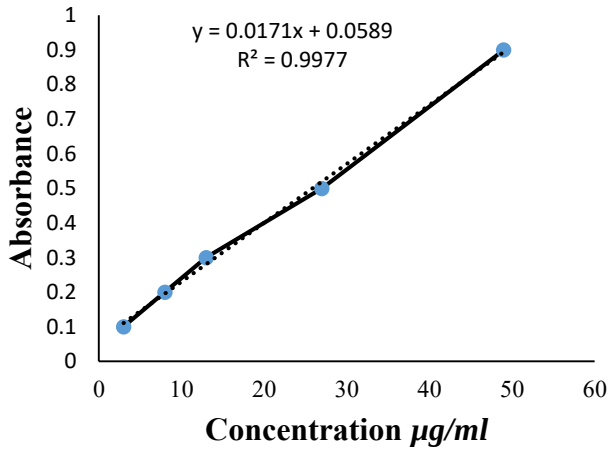


Figure 2.7: Ascorbic acid calibration curve.

Total anti-oxidant capacity

For the evaluation of total antioxidant capacity of selected plants extracts, phospho molybdenum method is used. In this assay Mo (VI) is converted to Mo (V) by antioxidants of extracts. By estimating total antioxidant capacity, it was observed that methanolic leaves extracts of *C. fistula* (30.95±0.35) and *F. racemosa* (21.93±1.41) µg/mg possess maximum antioxidant activity. The antioxidant power of *F. racemosa* and *S. cumini* is in the range of standard ascorbic acid (Table 2.4, Figure2.6).

Table 2.4: Total antioxidant capacity of different solvents extracts of selected plants

Conc.(µg/ml)	**Extracts solvents**	**Absorbance of extracts (Mean ± SD)**		
		FR (*µg/ml*)	CF (*µg/ml*)	SC (*µg/ml*)
1000	Me	21.93 ± 1.41^{bc}	30.95 ± 0.35^{a}	19.53 ± 2.14^{c}
	AQ	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.

Phytochemical screening of crude extracts

Qualitative Phytochemical Screening

Different phytochemical tests were used for the qualitative phytochemical screening of methanolic and aqueous leaves extracts of *F. racemosa, C. fistula* and *S. cumini*. Results of phytochemical tests showed that methanolic leaves extracts of all the selected plants are rich in antioxidant compounds as compared to their aqueous extracts.

Flavonoids are extracted from almost all plants but most abundant in *C. fistula* methanolic extracts. Few compounds like proteins and alkaloids are present in aqueous but not in their methanolic extracts (Table 2.5). Confirmed phytochemicals of methanolic and aqueous extracts of selected plants were graphically presented.

Table 2.5: Preliminary qualitative biochemical analysis of aqueous and methanolic extracts of selected plants

S.N	Chemical Compounds	Test name	Observations	*Ficus racemosa*		*Casia fistula*		*Syzigium 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	Ferric chloride	Bluish color formed	+	+	+	–	+++	–
5	Flavonoids	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.

Quantitative phytochemical analysis

Total phenolic and flavonoids contents

In present study aqueous and methanolic extracts of selected plants were evaluated for the total phenolic and flavonoids contents determination. Amongst the different solvents and plants extracts, maximum total phenolic (32.50±0.00) and flavonoids (40.00±1.00) *µg/ml* contents was in methanolic extract of *C. fistula,* while least amount of total phenolic contents were in aqueous extract of *F. racemosa* (25.00±1.73) and *S. cumini* (21.90±1.73) *µg/ml.* Total flavonoids lowest content was observed in aqueous extract of *F. racemosa* (26.67±0.58) *µg/ml* (Table 1.6).

Table 2.6: Quantitative phytochemical screening (TPC and TFC) of aqoueous and methanolic extracts of selected plants

S.No	Plants names	Solvents	TPC(µg/ml)	TFC(µg/ml)
1	*F. racemosa*	ME	28.33 ± 2.08^{ab}	32.57 ± 2.00^{ab}
		AQ	25.37 ± 1.73^{ab}	26.76 ± 0.58^{b}
2	*C. fistula*	ME	32.50 ± 0.00^{a}	40.00 ± 1.00^{a}
		AQ	25.00 ± 1.73^{ab}	32.63 ± 2.00^{ab}
3	*S. cumini*	ME	26.78 ± 1.73^{ab}	30.73 ± 1.15^{ab}
		AQ	21.90 ± 1.73^{b}	29.20 ± 1.15^{b}

Same superscript letter values are not significantly different at $p \geq 0.05$.

DISCUSSION

Eimeria is a protozoan parasite that cause the enteric disease coccidiosis in all major livestock species. The consequences of infection include malabsorption, enteritis and in severe cases for some *Eimeria* species, mortality, compromising economic productivity and animal welfare (Blake *et al*., 2020). 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*., 2019). Therefore, the current study was aimed to explore anticoccidial and antioxidant activities of selected plants *i.e*., *Ficus racemosa*, *Cassia fistula* and *Syzigium cumini*. 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. Similarly, Moriasi *et al*., (2020) revealed that methanol had a higher extractive value (38.92) of *Piliostigma thonningii* than water (18.27). Regarding anticoccidial activity 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 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 (20.2) and flavonoid (22.81)mgQE/g contents. According to Kaneria *et al*., (2013) antioxidant activities of extracts are highly related to their total phenols and flavonoids contents.

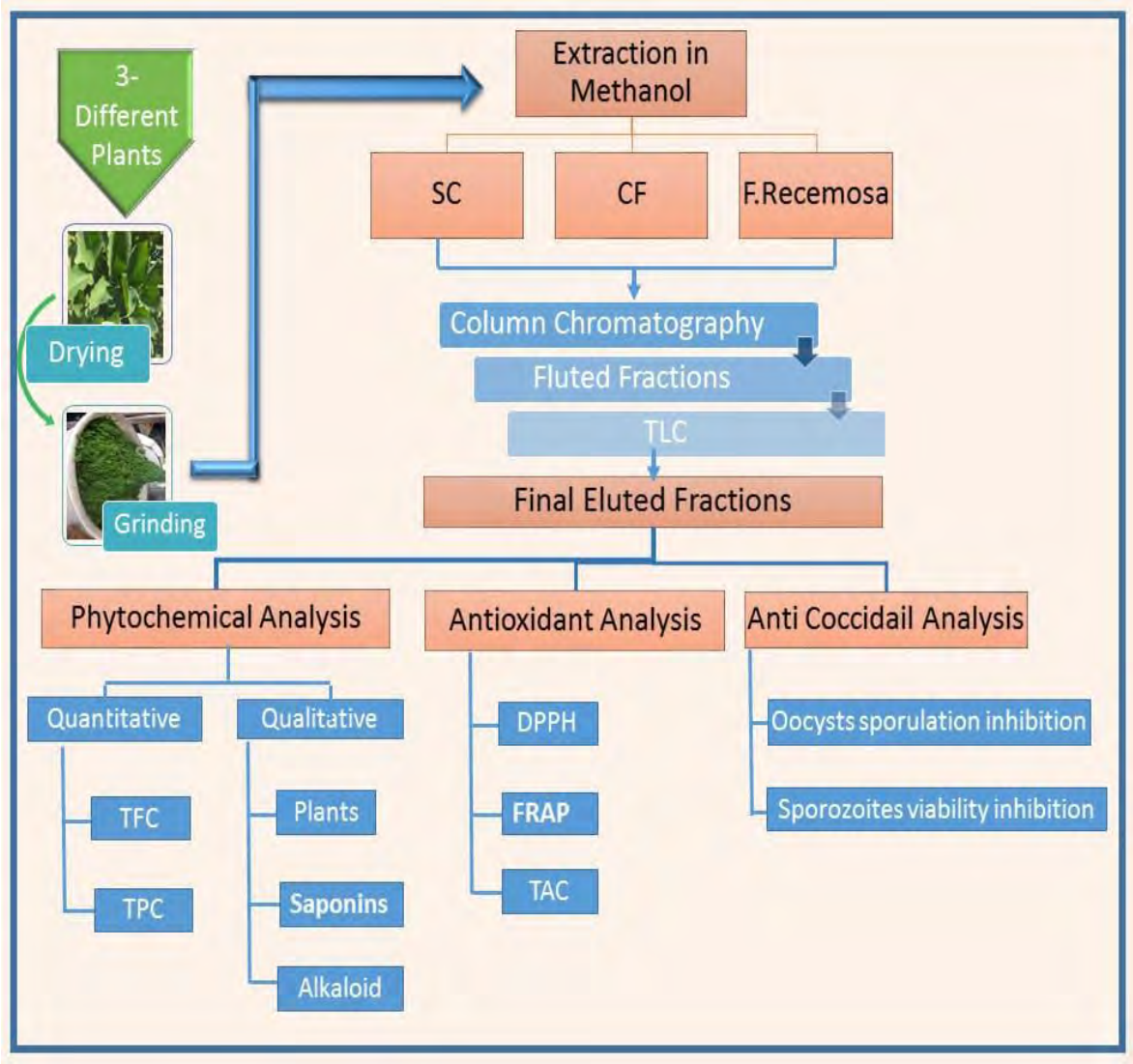
Conclusion

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.

Chapter 3

Title: Fractionation of crude methanolic extracts of selected plants and evaluvation of their antioxidant and *in vitro* anticoccidial potential

Graphical Abstract



ABSTRACT

The poultry industry is the world's most economically important industry and is badly affected by coccidiosis. *Eimeria* species develop resistance to drugs. Therefore, the present study was aimed at fractionating *F. racemosa*, *C. fistula* and *S. cumini* crude extracts for the identification of anticoccidial active fractions. Column chromatography and thin layer chromatography were used to fractionate the samples. For the evaluation of *in vitro* anticoccidial potential, sporozoites viability inhibition an oocysts sporulation inhibition assays were used. Preliminary qualitative, quantitative phytochemical screening and antioxidant potential of each fraction was performed. Each fraction antioxidant potential was measured by total antioxidant capacity estimation, ferric reducing antioxidant power and DPPH radical scavenging assays. After fractionation of the crude extracts, a total of 18 fractions from *F. racemosa*, 23 fractions from *C. fistula* and 19 fractions from *S. cumini* were eluted with different *Rf* values. The maximum oocysts sporulation inhibition (67.11±2.18%) was shown by F-19 of *F. racemosa* at 30mg/ml against *E. tenella*. Lowest efficacy (3.31±2.37%) was shown by fraction 7 at a concentration of 2.5 mg/ml against *E. mitis*. In *C. fistula* fractions crude extract, the maximum sporulation inhibition efficacy (69.11±2.18) and (69.56±1.14) % were shown by F-22 at a concentration of 30mg/ml against *E. tenella* and *E. necatrix* respectively. While the lowest efficacy (4.56±2.05%) was shown by F-4 at a concentration of 2.5*mg/ml* against *E. mitis*. Among the eluted fractions (n = 19) of *S. cumini* crude extract, F-19 inhibits maximum oocyst sporulation of *E. necatrix* at a concentration of 30mg/ml, showing sporulation inhibition of (76.56±1.14%). While the least oocysts sporulation inhibition was recorded in F-9 at a concentration of 2.5mg/ml against *E. necatrix* having a sporulation inhibition of (2.03±2.38%). Among all eluted fractions highest sporozoites viability inhibition percentage (72.56±1.23%) was shown by F-16 of *S. cumini* at 30*mg/ml* concentration against *E. mitis.* In all eluted fractions, maximum DPPH radical scavenging inhibition was shown by F-15 of *F. racemosa* with the lowest IC_{50} value (10.57±1.08) *µg/ml*. The range of this IC_{50} value is very close to the IC_{50} value of standard ascorbic acid (6.47±2.78) µg/ml. The maximum total antioxidant capacity (45.41±1.55) µg AAE/mg was possessed by F-15 of *F. raccemosa*. This indicates that these fractions possess high antioxidant power because their TAC is in close range to the antioxidant power of standard ascorbic acid (45.32±3.01). The highest ferric reducing power was recorded in F-19 (3.7±2.5) µg AAE/mg of *S. cumini*

at a concentration of 600µg/ml. Different fractions of all selected plants possess the maximum tested phytochemicals. Among all the eluted fractions of selected plants, maximum total phenolic (120.00±2.13) µgGAE /mg and flavonoid contents (125.57±1.56) µgGAE /mg were recorded in F-22 of *C. fistula*. According to the findings of the current investigation, bioactive fractions of methanolic extracts of certain plants have the highest anti-oxidant and anticoccidial activity because they include pharmaceutically significant phytochemicals. Further study is needed to identify and isolate anticoccidial active chemicals from these fractions that may be utilized to produce drugs to treat coccidiosis.

INTRODUCTION

Poultry industry is an important agricultural sector that supports economic development. However, their productivity is decreased due to a parasitic disease coccidiosis coccidiosis caused by a protozoan parasite *Eimeria,* (Lawan *et al*., 2016). Seven species of the apicomplexan parasite *Eimeria* have been identified as the primary causes of coccidiosis in chicks (Quiroz -Castaneda and Dantan-Gonzalez., 2015). This parasite's oocysts enter the host through the mouth, infecting various parts of the gut and caecum, where they reproduce. Cause bloody diarrhea, poor absorption, decreased weight gain, dehydration and ultimately death in chicks (Pawestri *et al*., 2019). Seventy percent of commercial poultry breeds are affected by this deadly illness in developing countries like Pakistan (Abbas *et al*., 2017). The use of various control strategies is reported in literature, such as anticoccidial medications and vaccinations (Kadykalo *et al*., 2018). However, several limitations, such as the expense of vaccine manufacture (Adhikari *et al*., 2020) and the development of drug resistance in *Eimeria* species are of grave concern (Karavolias *et al*., 2018). In order to prevent bacterial infections and boost development, antimicrobial feed additives known as antibiotic growth promoters (AGP) have been utilized in chicken production. However, there is growing demand to eliminate AGP from poultry production. In the case of AGP-free poultry production, several natural feed additives have beneficial impacts on the intestinal health of broiler chicks and enhance growth performance (McKnight *et al*., 2019). The use of natural products to manage coccidiosis is one such alternative strategy that is needed in the current situation. Natural products are being considered as possible viable alternative substituents. Although there has been significant progress in recent years, but more research is still needed on edible plants and their compounds can be considered a viable alternative to the current anti-coccidial strategies. This is because of their safety, effectiveness, and the mechanisms of their modes of action (Sharman *et al*., 2010). Anti-protozoal capabilities of more than 1200 plants have been reported. (Muthamilselvan *et al*., 2016; Willcox, 2004).

Only 20 herbal plants have been investigated thus far for their anti-coccidial properties (Orengo *et al*., 2012; Youn and Noh; 2001). At least four plant-based products, including Cocci-Guard, are now marketed and can be added to chicken and/or other animals' feed as anticoccidial feed additives. Antioxidants are chemicals that

interact with free radicals to stop their oxidative reaction and guard against cellular harm. Free radical oxidative species are produced during the host's cellular immunological response to an invasion by an *Eimeria* species (Allen *et al*. 1997) playing a significant protective role against parasite infections, but very excessive concentrations can cause cytotoxicity and tissue damage, which can worsen infection pathology. Because of this, a severe coccidial infection involves pathogenic oxidative stress and altered ecological oxidative balance, which show up as weight growth, changes in the caecum, and feed conversion ratio (Georgieva *et al*., 2006). The anticoccidial activity of a herbal substance is due to its antioxidant function (Alhotan and Abudabos, 2019).

In fact, several researchers have reported that plants contain a range of phytochemical substances, such as polyphenols and alkaloids, which have the capacity to interfere with the cell membrane or cytoplasm of parasites and cause their death. (Muthamilselvan *et al*., 2016). Therefore, to isolate and purify bioactive phytochemicals for the purpose of synthesizing useful antibacterial, anticancer, and antibiotic compounds, crude plant methanolic extract is exposed to various chromatographic methods such as column chromatography and thin layer chromatography (Pratik and Thakur., 2020).

The technique of column chromatography is used to separate specific chemical compounds from mixtures of chemical compounds. It is frequently applied for preparative purposes on scales ranging from micrograms to kilo grammes. The main benefit of column chromatography is that the stationary phase used in the procedure is very inexpensive and easily disposed away (Abdulhamid *et al*., 2017). The latter stop cross contamination and recycling-induced stationary phase deterioration. Thin-layer chromatography (TLC) is a technique that is often used in synthetic chemistry to identify chemicals, evaluate their purity, and monitor the course of a reaction. In TLC it is also possible to change the solvent. It is more quick and takes much less of the sample than column chromatography. A thin, homogenous coating of silica gel or alumina is applied on a piece of glass, metal, or rigid plastic to generate a thin layer chromatography. The mobile phase consists of a suitable liquid solvent or solvent mixture (Jonathan *et al*., 2007).

A well-known medicinal plant *F. racemosa* (family: Moraceae) native to South-East Asia, India, and Australia was chosen for the current study. Several parts of this

plant contain a large number of pharmacologically useful substances (Mohiuddin and Lia., 2020). *F. racemosa* contain alkaloids, phenolic acids, coumarins, flavonoids and etc. Phenolic compounds, vitamin C and flavonoids are among the non enzymatic one. There are four different types of enzymes present *i.e*., ascorbate oxidase, ascorbate peroxidase, catalase, and peroxidase. Gallic acid and ellagic acid are the phenolic components in this mixture (Keshari *et al*., 2016). The *F. racemosa* plant has a variety of medical uses. For example, the leaves of the plant are used to cure diarrhea, while the fruit and bark act as astringents to treat hematuria and menorrhagia. The fluid of the tree roots is said to be tonic when used for longer period of time. The sap is a well-known therapy for gonorrhea in Bombay and is utilized topically to treat mumps and other inflammatory glandular enlargements. To cure tonsillitis, their root is eaten (Mohiuddin and Lia., 2020). Pharmacologically this plant is also important by working as antibacterial, antimicrobial and analgesic agent (Collee *et al*., 1996).

A well-known plant found in deciduous forests rising up to 1300 meters in the outer Himalaya is called *Cassia fistula*, often known as amaltas or Golden Shower (Neelam *et al*., 2011). It is a member of the Fabacae family. It can grow anywhere, even on trap, rock, and stone soil as well as in poor, shallow soil. In addition to other Asian countries including India, Philippines, Hong Kong, China, Mexico, Africa, South Asia, Malaysia, Indonesia, and Thailand, it is widely grown across Bangladesh. Since ancient times, the whole plant has been used as an ingredient in several remedies to cure a variety of disorders due to its therapeutic characteristics (Bhalodia and Shukla, 2011). Due to its wide range of pharmacological properties like anti microbial, anti-fertility, anti inflammatory, antioxidant and etc, it has a wide range of therapeutic applications. Its leaves and bark are used to treat skin diseases, while its roots are a diuretic and can be used to treat ulcers, tubercular glands, heart issues, and skin conditions. Its fruit pulp is used as a mild laxative for a range of gastrointestinal problems. Leprosy, gastrointestinal issues, and fever may all be cured with its blooms. Its seeds may have laxative, relaxing, and antipyretic properties. The plant is an excellent source of carbohydrates, linoleic, oleic, and stearic acids as well as tannins, glycosides, and flavonoides. It also contains glycosides, free rhein, sennosides A and B, isofavone oxalic acids, oxyanthraquinones derivatives, tannins, B-sitosterol, arginine, leucine,

astringent compound, glutten, fistulic acids, and subordinates of flavonid-3-ol (Saeed *et al*., 2020).

A potential source of medicinal polyphenols was studied using the *Syzygium cumini* seeds extracts (SCSE). One of the medicinal plants that belongs to the Myrtaceae family abundantly found in Asia. Due to its antibacterial, anti-diabetic, anti-inflammatory, etc. properties, *Syzygium cumini* seeds have been used for many medical purposes throughout history (Swami *et al*., 2012). The extracts of seed contain a variety of useful chemicals, including gallic acid (GA), jambosine, ellagic acid and etc. In the pharmaceutical and food sectors, it can be used as a dietary antioxidant booster (Balyan and Sarkar, 2016). *S. cumini* leaf extract has notable anti-inflammatory, anti-oxidant, and anti-glycation potential. It also has strong ability to inhibit the enzymes lipase and amylase. Additionally, the plant material was not harmful to the erythrocytes, favoring its usage as a therapeutic agent. This study's conclusions about *S. cumini's* biological and pharmacological capabilities may help researchers better understand how to use the plant to treat T2DM and associated complications (Franco *et al*., 2020). Since the previous several decades, numerous attempts have been undertaken to isolate and identify bioactive chemicals from medicinal plants, and their biological characteristics have been established. Many of these chemicals are now being created in laboratories for use in the pharmaceutical sector (Salihu and Usman, 2015). Consequently, the goal of the current work was to fractionate and examine antioxidant and anticoccidial active fractions from *C. fistula*, *F. racemosa* and *S. cumini* methanolic leaf extracts for the first time.

MATERIALS AND METHODS

Crude extract preparation

Methanolic extracts of the powdered leaves of *F. racemosa*, *C. fistula*, and *S. cumini* were prepared using the maceration process of extraction (Cedric *et al*., 2018). In the maceration extraction technique, 150gm of leaf powder was taken from three selected plants in separate conical flasks and permitted to soak in 500ml of methanol. The contents were kept for 2 days in conical flasks and shaken vigorously. After being stirred, the mixture was filtered at a constant temperature (45°C) using Whatman No. 1 filter paper. Solvent was fully evaporated using a rotary evaporator at 50rpm and 40°C. The methanolic extracts produced were kept in a refrigerator at 4°C in glass beakers covered with aluminium foil for further processing (Sultana *et al*., 2013; Ezeh *et al*., 2020).

Crude extract Fractionation

Column Chromatography

For the isolation of different fractions from crude methanolic extracts of selected plants, a cylindrical glass column (28×3 inches) was used and packed by the wet packing technique. At the base of the column, glass wool was used to prevent the flow of silica gel. A slurry of (300g) silica gel (0.063-0.200mm Merck) was prepared in 300ml of hexane and continuous stirring occurred. During stirring, a slurry of silica gel is poured into the glass column (Figure 3.1). continuously tapped the column to avoid bubble formation. The sea sand is added to avoid disturbance of the surface of the silica column (Kumar and Sachin, 2013).

Loading of sample and column treatment

The crude methanolic extract (18gm) of each selected plant leaves were mixed with silica gel to form a dry powder. Samples were added over sea sand in the column. A gradient elution technique was used to run the column throughout four different solvents i.e., n-hexane, ethyl acetate, chloroform and methanol in increasing order of polarity. Various ratios of the above mentioned solvents were used in different concentrations for the isolation of fractions containing different compounds (Bajpai and Kang, 2011).

Figure 3.1. Different bands in packed column due to movement of mobile phases.

Thin layer chromatography

The fractions eluted by column chromatography were subjected to thin layer chromatography (TLC) using silica coated TLC plates (15×8cm) (Merck, Mumbai). The start line at 1.5cm on the TLC plate was drawn by pencil. Each fraction (0.1µl) was spotted carefully on line. Then each fraction TLC plate was immersed in its respective solvent until the development of color spots. Plates were examined by UV light after drying.

The spots' retention factor (*Rf*) value was calculated using the following formula;

$$Rf = \frac{Distance\ traveled\ by\ solute}{Distance\ traveld\ by\ solvent}$$

Different fractions having the same *Rf* values were mixed (Table 3.1, 3.2 and 3.3). Solvents were evaporated and crude fractions were used for further evaluation (Ahmad *et al*., 2016).

Phytochemicals screening of isolated fractions

Qualitative Analysis

The qualitative phytochemical analysis was conducted by using traditional methods for the presence of bioactive compounds in each fraction according to the methods of Shabrina *et al*., (2018).

Quantitative analysis

Total flavonoids contents (TFC)

The colorimetric method with slight modification was used to calculate the quantity of total flavonoids. Quercetin was used as standard (Kaneria *et al*., 2009). 20µl of each fraction (4 mg/ml) along with control quercetin (1mg/ml) were collected and incubated for 30 minutes at 37 °C in a 96-well plate. Then 10µl of aluminium chloride (10%),10µl of potassium acetate (98.15g/l) was added and raising the final volume with distilled water up to 200µl. Microplate readers were used to determine the absorbance at 405 nm, and the results were analyzed as gQE/mg extracts in triplicate.

Total phenolic contents (TPC)

Total phenolic contents were determined with Folin-Ciocalteu reagent technique with slight modification. Each fraction (1mg) was dissolved in 1ml of its mother solution. From each crude sample, 200µl was taken in separate test tube and 10% Folin Ciocalteu reagent (1.5ml) was added. Then in dark place all the test tubes were kept for 5 minutes. Then all the test tubes were kept for 5 minutes in a dark place. Then 5% Na_2CO_3 (1.5ml) was added. After mixing all the test tubes were again kept in in dark place for 2 hours. Each sample absorbance was measured at 750nm wavelength by using UV spectrophotometer (Jagadish *et al*., 2009).

Antioxidant assays

DPPH radical scavenging assay

The method suggested by Tai *et al*., (2011) was used to measure the free radical scavenging activity. In 96 well plates, different concentration of each fraction (5,10, 15 and 20 µg/ml) were used. To get the final concentration of 200 µl, DPPH reagent was added to each row of wells. Ascorbic acid was used as control. A microplate reader was used to measure absorbance at 630 nm following an hour of incubation at room temperature. The percent radical scavenging percentage was calculated by following formula;

$$Radical\ scavenging\ (\%) = [(A0\text{-}A1/\ A0) \times 100\%]$$

A1 is the absorbance of the sample extracts, while *A0* is the absorbance of the control. The antioxidant activity of each sample was expressed in terms of IC_{50}. IC_{50} values are expressed as *µg AAE/mg* of extracts.

Ferric reducing antioxidant power assay (FRAP)

The ferric reducing power of each fraction was measured by using the standard procedure (Patil *et al*., 2009). In distilled water, different concentrations of each fraction and ascorbic acid (100, 300, and 600 ug/ml) were prepared (1ml). Each fraction was mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and potassium feericyanide (1%) solution. The mixture was then placed in an incubator at 50°C for 20 minutes. 2.5 ml of tri chloroacetic acid (10%) was added after incubation. At a speed of 3000 rpm, the mixture was centrifuged for 10 minutes. The supernatant was collected, distilled water (2.5ml) and ferric chloride (0.1%w/v) was added. A spectrophotometer was used to measure their absorbance at 700 nm.

Total antioxidant capacity estimation (TAC)

The phospho molybdenum technique was used to evaluate the fractions overall antioxidant capacity (Sakat *et al*., 2010). This method transforms molybdenum (VI) to molybdenum (V) by forming a green phosphate/Mo (V) complex (Jafri *et al*., 2014). 20µl of each test sample (4 mg/ml) were mixed with 180µl of phospho molybdenum reagent. Then incubated in a water bath for 90 minutes at 95 °C. At room temperature samples were cooled after incubation, thereafter transferred to 96 well plates. As a control ascorbic acid was used. A microplate reader was used to measure absorbance at 630nm. Results have been expressed as ascorbic acid *µg/ml* of extract.

***In vitro* anticoccidial assays**

Collection of samples and identification of *Eimeria* species

From veterinary research institute (VRI) Peshawar, coccidiosis suspected chick guts were collected. Oocysts of different *Eimeria* species from gut contents were collected and stored in a 2.5% solution of $K_2Cr_2O_7$ (Wajiha *et al*., 2018). Different species of *Eimeria* were identified on the basis of oocysts size, morphology and site of infection (Gadelhaq *et al*., 2018).

Sporulation inhibition of oocysts

All fractions sporulation inhibition efficacy was evaluated in petri dishes. 1500 unsporulated oocysts and 2ml of each concentration of every fraction were added to different petri dishes. For 48 hours at 28°C all of the petri dishes were incubated. In each petri dish the no of sporulated and unsporulated oocysts were counted. In each petri dish the percentage of sporulation was calculated by counting the no of sporulated oocysts in a total of 100 oocysts. Amprolium (1.25mg/ml) and DMSO were taken as

positive and negative control respectively. For the calculation of sporulation inhibitory percentage, the following formula was used (Cedric *et al*., 2018).

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

Sporozoites viability inhibition assay

In the sporozoite viability inhibition assay, first the sporozoites were liberated from sporulated oocysts by following the methods of Cedric *et al*., (2018). To the different petri dishes, 1500 sporozoites and 2ml of each concentration (2.5, 5, 10, 20 and 30 mg/ml) of fractions were added. Amprolium (1.25mg/ml) and DMSO were used as positive and negative control respectively. For 48 hours at 28°C, the complete set up was incubated. By counting the amount of viable sporozoites out of a total of 100 sporozoites, the viability inhibition percentage of sporozoites was calculated. For the determination of viability inhibitory percentage of sporozoites, the following formula was used;

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

Statistical Analysis

The collected data was analyzed by using statistical software for social sciences (SPSS) version 23. The data were shown with their mean and standard deviation in triplicate. T-tests are applied to compare mean values. A simple regression analysis for the Pearson correlation coefficient of TP, TF, and antioxidant activities was determined. When $P<0.05$, values were considered significant.

RESULTS

Crude extract fractionation and *Rf* values

Fractionation of the methanolic extracts of *F. racemosa*, *C. fistula* and *S. cumini* was done by column chromatography using silica gel (0.063-0.200 mm Merck). During column chromatography increasing order of polarity from non-polar to polar mobile phase was followed for elution of fractions. From *F. racemosa*, about 67 fractions with different polarities, each approximately 50ml in volume, were collected. Based on the TLC profile, fractions with similar *Rf* values were combined and crystallized. After mixing of the same *Rf* value fractions, a total of 18 fractions with different *Rf* values were finally obtained (Table 3.1). A total of 52 fractions, each approximately 50ml in volume with different polarities, were collected from the methanolic crude extract of *C. fistula*. Fractions of the same *Rf* values were combined and crystalized on the basis of TLC profiling. Finally, 23 fractions of different polarities and *Rf* values were obtained (Table 3.2). From the crude methanolic extract of *S. cumini* after fractionation, 67 fractions altogether were collected. On TLC profiling, same *Rf* values fractions were mixed and, at the end, 19 fractions having different polarity and *Rf* values were obtained (Table 3.3; Figure 3.2).

Figure 3.2: Final eluted fractions of selected plants crude extracts.

Qualitative phytochemical screening

The final obtained 18 fractions of *F. racemosa*, 23 fractions of *C. fistula* and 19 fractions of *S. cumini* leaves methanolic extracts were put through a series of qualitative phytochemical assays to check for alkaloids, carbohydrates, saponins, flavonoids, phenols, terpenoids, glycosides and proteins as per standard procedures. From the results, it was evident that fraction 1-9 of *F. racemose* showed positive results for phenols, carbohydrates and saponins, while fractions 10-13 showed positive results for phenols, flavonoids, alkaloids, proteins and glycosides. It was also evident that the fractions from 14-19 showed positive results for phenols, flavonoids, carbohydrates,

proteins, and alkaloids. The highest levels of phenols and flavonoids were found in fractions 15 and 19 (Figure 3.3).

Among the 23 fractions of *C.fistula*, fractions 1-3 showed positive results for alkaloids, carbohydrates, phenols, flavanoids , terpenoids, glycosides, and proteins. F-4 has all the tested phytochemicals except alkaloids. In F5-13, all the tested phytochmecials are present. In the remaining fractions, F-14 and F-18 have very low amounts of proteins and glycosides respectively. Maximum phenols and flavanoids were present in F-20 and F-22 (Figure 3.4). *S.cumini* eluted 19 fractions also possessed the maximum number of tested phytochemicals. The highest levels of phenols and flavanoids were found in F-6, 13, 16, and 19. F-18 tested negative for proteins. Maximum saponins were recorded in F8, 16 and 19 (Figure 3.5).

Quantitative analysis

To quantify the overall phenolic and flavonoid content of each of the selected plant fractions, quantitative analysis was carried out. Among the 19 fractions of *F. racemosa*, the highest amount of total phenolic content (71.03±1.09) in F-19, followed by F-15 (53.27±2.93) µgGAE/mg and total flavonoids (72.41±2.74) in F-15 followed by F-19 (67.21±2.69) µgQE/mg was observed. While lowest amount of total phenolic (07.09±2.31) in F-7 and total flavonoids in (15.04±1.22) in F-4 was observed (Figure 3.6).

In 23 fractions of *C. fistula*, the maximum total phenolic content (120.00±2.13) was recorded in F-22 followed by F 20(112.50±1.06) µgGAE /mg. while their lowest amount was observed in F-2 (2.50) followed by F-1(7.50) and F-4 (7.89). Total flavonoids contents were present in maximum amount in F-22 (125.57±1.56) followed by F-20(122.07±3.45). Whereas the lowest amount of total flavonoids contents was recorded in F-3 (7.50±2.67) µgQE /mg (Figure 3.7).

Among the 19 fractions of *S. cumini* the maximum phenolic content was present in F-19 (97.5±2.05) followed by F-16(95.00±1.56) *µgGAE /mg*. while the lowest amount was found in F-4(2.50±1.67) followed by F-9 (7.50±3.27) *µgGAE /mg*. In *S. cumini* 19 fractions the maximum total flavonoids contents were recorded in F-19 (112.5±2.13) followed by F-16(97.5±1.56) *µgQE /mg*. While the least amount of total flavonoids contents was recorded in F-5 (5.05±1.56) and F-8 (5.67±2.34) (Figure 3.8).

Table 3.1: Column chromatography, observations on TLC and *Rf* values of the methanolic extract of *F. racemosa* fractions

S. No	Solvent system	Ratio	Fractions	Volume (ml)	Observations on TLC	Final fractions	Rf Values
1	Hexane	100	1_6	50,48,50,44,50,50,	F1_F5: No spot	-	-
					F7: single spot	-	-
2	Hexane: Ethyl acetate	80:20:00	7_9	47,50,50	F7_F9: 3 similar spots	2	0.59
3	Hexane: Ethyl acetate	60:40:00	10_12	48,48,45	F10:Single spot	3	0.37
					F11 and 12:No spots	-	-
4	Hexane: Ethyl acetate	40:60	13_15	44,45,45	F13_15: 3 similar spots	4	0.44
5	Hexane: Ethyl acetate	20:80	16_19	45,50,50,44	F16 and 19: 4 similar spots	5	0.39
6	Ethyl acetate	100	20_24	48,45,50,43,50	F20: Single spot	6	0.37
					F21-24: 3 similar spots	7	0.25
7	Ethyl acetate: Chloroform	80:20:00	25_27	50,44,45	F25: No spot	-	-
					F26-27: 2 similar spots	8	0.38
8	Ethyl acetate: Chloroform	60:40:00	28_33	46,48,45,50,47,44	F28: Single spot	9	0.41
					F29_33: 5 similar spots	10	0.17
9	Ethyl acetate: Chloroform	40:60	34_37	48, 45,44,50	F34_37: 4 similar spots	11	0.27
10	Ethyl acetate: Chloroform	20:80	38_39	50,48	F38_39: 2 spots	12	0.26
11	Chloroform	100	40_47	48,49,46,50,43 ,44,48,50	F40-43: No spot	-	-
					F44-47: 4 similar spots	13	0.28
12	Chloroform: Methanol	80:20:00	48_53	50,48,44,48,50,48	F48-49: single spot	14	0.25
					F50 and 53:3 similar spots	15	0.54
13	Chloroform: Methanol	60:40:00	54_57	48,44,46,50	F54: No spot	-	-
					F55-57: 3 similar spots	16	0.47
14	Chloroform: Methanol	40:60	58_62	50,48,44,50,47	F58: No spot	-	-
					F59-62:4 similar spots	17	0.29
15	Chloroform: Methanol	20:80	63_65	44,50,48	F63-65: 3 similar spots	18	0.42
16	Methanol	100	66_67	50, 50	F66-67: 2 similar spots	19	0.57

Table 3.2: Column chromatography, Observations on TLC and *Rf* values of the methanolic extract of *C. fistula* fractions

S. No	Solvent system	Ratio	Fractions	Volume (ml)	Observations on TLC	Final fractions	Rf Values
1	Hexane	100	1_4	48, 45, 50,44	F1-F3: No spot on TLC	-	-
					F4: single spot	1	0.47
2	Hexane: Ethyl acetate	80:20:00	5_7	50,48,46	F5: Single spot	2	0.59
					F6, F7: 2 Similar spots	3	0.44
3	Hexane: Ethyl acetate	60:40:00	8_9	50, 50	F8_9:Single spot	4	0.72
4	Hexane: Ethyl acetate	40:60	10_13	48,50,45,47	F10 and 11:No spots	_	_
					F12: 2 spots	5,6	0.57,0.62
					F13: No spot	_	_
5	Hexane: Ethyl acetate	20:80	14_15	50,50	F14 and 15: 2 similar spots	7	0.6
6	Ethyl acetate	100	16_19	46,44,48,50	F16: Single spot	8	0.42
					F17,18 and 19: 3 similar spots	9	0.54
7	Ethyl acetate: Chloroform	80:20:00	20_21	44,47	F20 and 21: 2 similar spots	10	0.38
8	Ethyl acetate: Chloroform	60:40:00	22_25	48,48,50,48	F22_25: 4 similar spots	11	0.25
9	Ethyl acetate: Chloroform	40:60	26_30	50,50,44,48,50	F26_30: 5 similar spot	12	0.42
10	Ethyl acetate: Chloroform	20:80	31_34	45,48,50,48	F31_34: 4 similar spots	13	0.36
11	Chloroform	100	35	50	F35: Single spot	14	0.28
12	Chloroform: Methanol	80:20:00	36_38	48,44,50	F36: single spot	15	0.34
					F37 and 38:2 similar spots	16	0.41
13	Chloroform: Methanol	60:40:00	39_41	48,50,46	F39: single spot	17	0.57
					F40 and 41: 2 similar spots	18	0.47
14	Chloroform: Methanol	40:60	42_47	50,44,46,48,48,50	F42_47: 6 similar spots	19	0.29
15	Chloroform: Methanol	20:80	48_50	48,44,50	F48 and 49:2 similar spots	20	0.42
					F50: single spot	21	0.37
16	Methanol	100	51_52	50,44	F51 and 52: 2 similar spots	22	0.21
					F53:single spot	23	0.14

Table 3.3: Column chromatography, Observations on TLC and Rf values of the methanolic extract of *S.cumini* fractions

S. No	Solvent system	Ratio	Fractions	Volume (ml)	Observations on TLC	Final fractions	*Rf* Values
1	Hexane	100	1_7	48,50,44,47,43,50,45	F1-F3: No spot on TLC	-	-
					F4-F7: 4 similar spots	1	0.45
2	Hexane: Ethyl acetate	80:20:00	8_11	44,50, 45,48	F8-F9: 2 similar spots	2	0.47
					F10- F11: No spot	_	_
3	Hexane: Ethyl acetate	60:40:00	12_17	50,48,50,44,47,50	F12_15:4 similar spots	3	0.39
					F16-17: No spot	_	_
4	Hexane: Ethyl acetate	40:60	18_21	50,48,45,50	F18 -21:4 similar spots	4	0.28
5	Hexane: Ethyl acetate	20:80	22_27	47,44,50,47,47,44	F22-26: No spot	_	_
					F27: Single spot	5	0.54
6	Ethyl acetate	100	28_31	46,48,50,44	F28-30: 3 similar spots	6	0.37
					F31: No spot	_	_
7	Ethyl acetate: Chloroform	80:20:00	32_35	48,45,50,50	F32 and 35: 4 similar spots	7	0.51
8	Ethyl acetate: Chloroform	60:40:00	36_39	50,48,48,44	F36_39: 4 similar spots	8	0.49
9	Ethyl acetate: Chloroform	40:60	40_44	50,50,46,44,50	F40_42: No spot	_	_
					F43-F44: 2 similar spots	9	0.37
10	Ethyl acetate: Chloroform	20:80	45_49	50,48,50,50,44	F45: No spot	_	_
					F46-49: 4 similar spots	10	0.28
11	Chloroform	100	50_53	50,50,44,47	F50-53: Single spot	11	0.63
12	Chloroform: Methanol	80:20:00	54_57	48,47,50,50	F54: single spot	12	0.26
					F55-57:3 similar spots	13	0.49
13	Chloroform: Methanol	60:40:00	58_59	44,48	F58-59: single spot	14	0.51
14	Chloroform: Methanol	40:60	60_61	50,50	F60_61: 2 similar spots	15	0.43
15	Chloroform: Methanol	20:80	62_63	44,45	F62: single spot	16	0.24
					F63: single spot	17	0.37
16	Methanol	100	64_67	45,47,50,50	F64-65: 2 similar spots	18	o.20
					F66-67:single spot	19	0.11

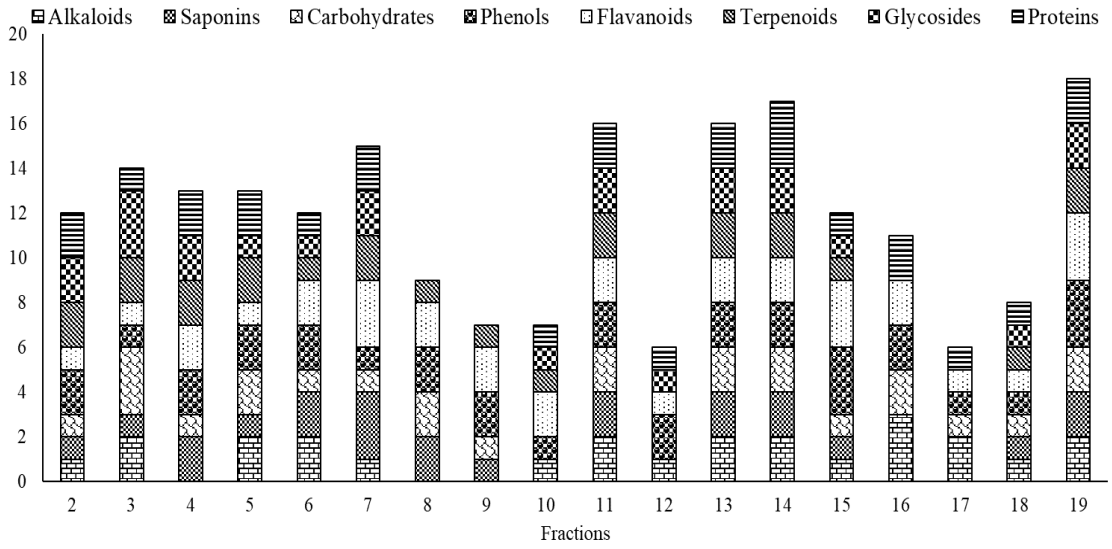


Figure 3.3: Qualitative phytochemical screening of isolated fractions of *F. racemosa* crud extract.

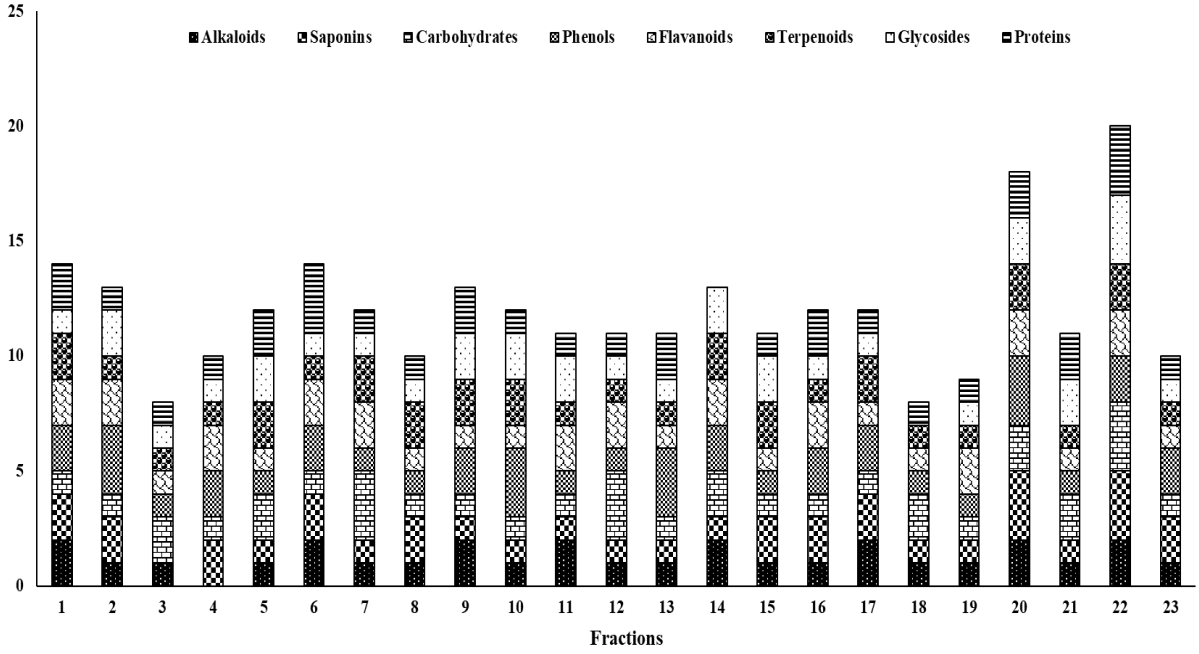


Figure 3.4: Qualitative phytochemical screening of isolated fractions of *C.fistula* crud extract

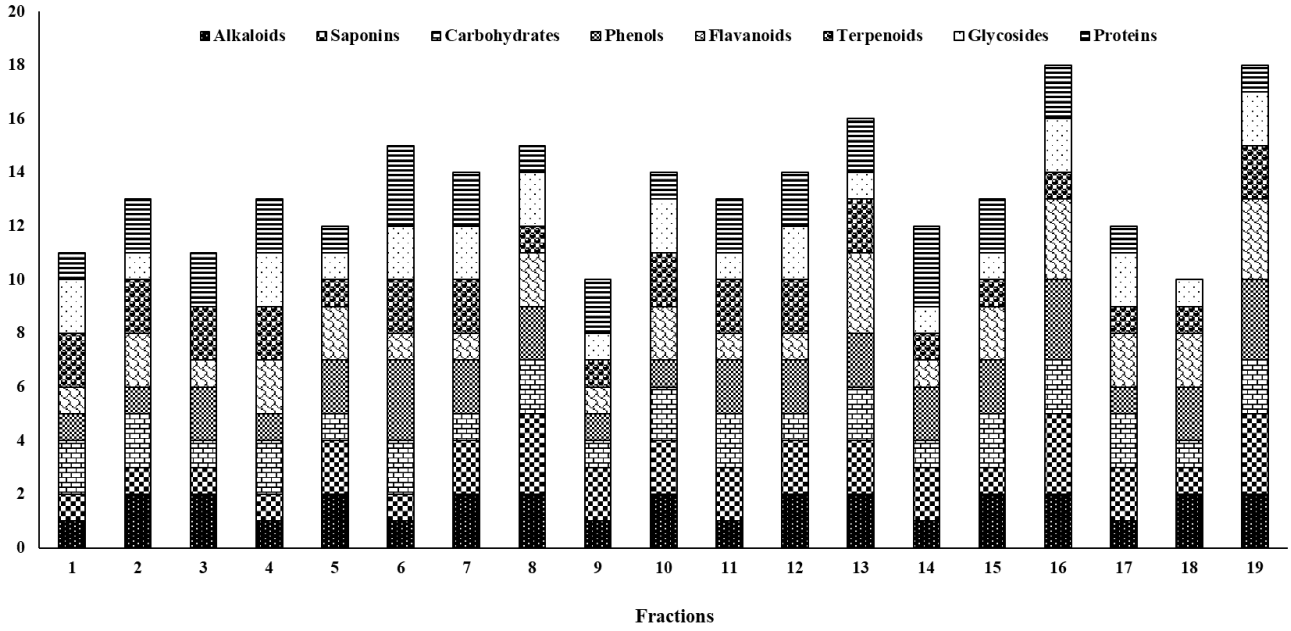


Figure 3.5: Qualitative phytochemical screening of isolated fractions of *S. cumini* crud extract.

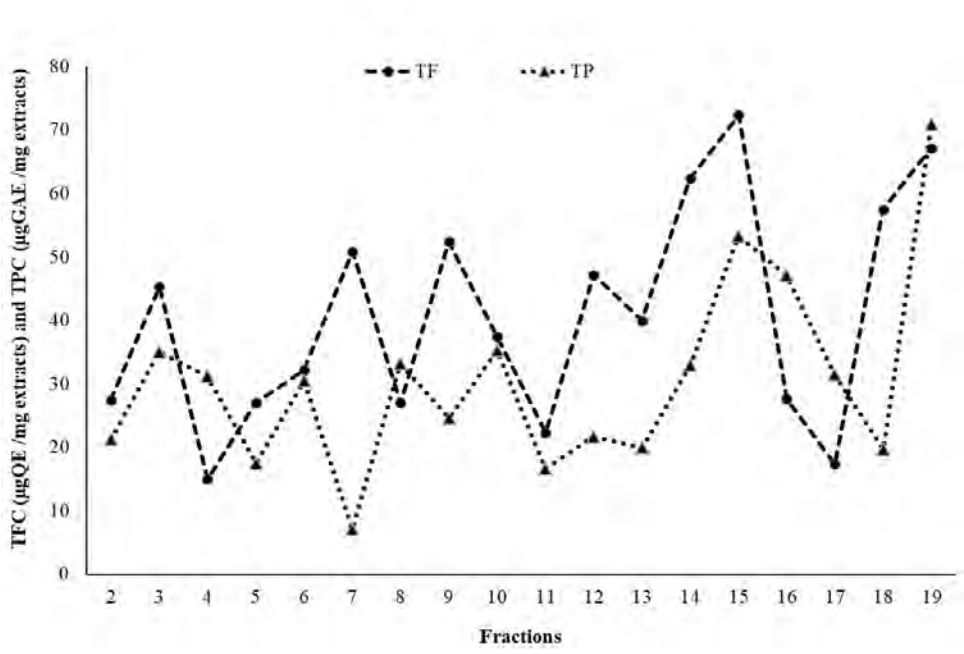


Figure 3.6: Total flavonoids and total phenolic contents of each fraction isolated from *F. racemosa* leaves extract.

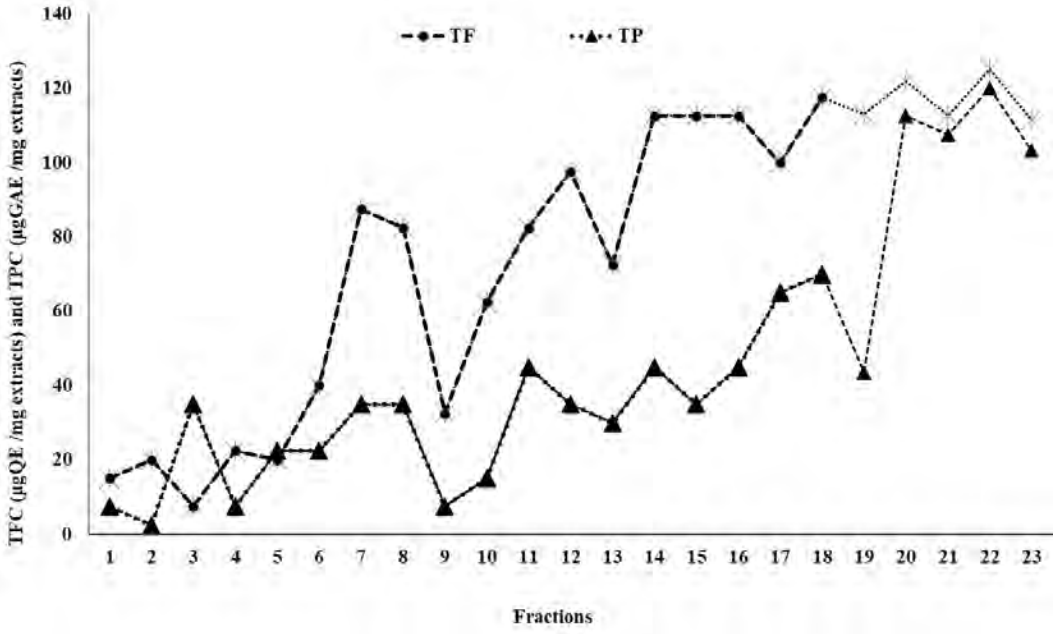


Figure 3.7: Total flavonoids and total phenolic contents of each fraction isolated from C. *fistula* leaves extract.

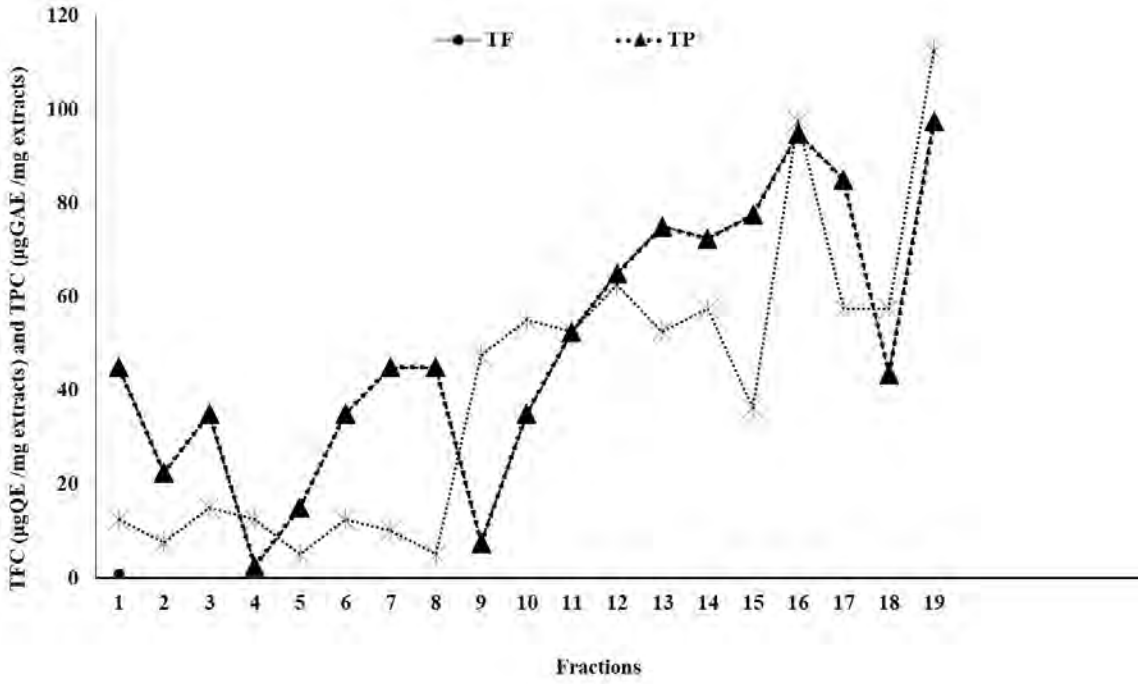


Figure 3.8: Total flavonoids and total phenolic contents of each fraction isolated from *S. cumini* leaves extract.

Antioxidant assays

Antioxidant activities of eluted fractions of were measured by DPPH, FRAP and TAC assays. Ascorbic acid was used as control for all of the assays.

DPPH assay

The DPPH test can be used to evaluate the natural substance's potential to donate electrons. A stable free radical called DPPH is capable of creating a stable diamagnetic compound from an electron or hydrogen radical. DPPH molecule will scavenge the electron or hydrogen that was provided by the natural substance. In the present study, almost all the fractions of selected plants have shown scavenging activities. Among the 19 fractions of *F. racemosa* crude extract, maximum inhibition was shown by F-19 and 15 with the lowest IC_{50} values (13.9±2.5) and (10.57±1.08) µg/ml respectively as compared to the standard ascorbic acid (Figure 3.9a and b).

Isolated 23 fractions of *C. fistula* possessed DPPH radical scavenging activity to some extent. The maximum scavenging ability with lowest IC_{50} values was shown by F-22 (12.28±2.56) and F-20(11.69±1.56)µg/ml approximately fall in the range of a strong antioxidant standard ascorbic acid (6.47) µg/ml (Figure 3.10a and b).

In case of *S. cumini* out of a total of 19 fractions, the maximum DPPH radical scavenging activity was shown by F-16 and F-19 having the lowest IC_{50} values as (15.78±2.34) and (15.47±1.56) µg/ml respectively. The range of these IC_{50} values is very close to the IC_{50} value of standard ascorbic acid (6.47±2.78) µg/ml. The weak DPPH radical scavenging activity was shown by F-3 and F-12 having the highest IC_{50} values (48.82±2.57) µg/ml among other fractions (Figure 3.11a and b). Overall, various fractions' ability to scavenge radicals increased in a concentration-dependent manner. The fact that all of the selected plants' active fractions eluted with methanol solvent suggests that the eluted chemicals were mostly polar.

Ferric reducing antioxidant power (FRAP)

The results of the present study indicate an increasing trend of reducing antioxidant power with increasing extract concentration in each fraction. Among the isolated fractions of *F. racemosa*, fractions 15 and 19 possess the maximum reducing power (2.9±2.17) and (2.13±1.00) *µg AAE/mg* at 600 *µg/ml* concentration respectively. That is slightly lower but statistically similar to standard ascorbic acid's reducing capability (3.9±1.04) *µg AAE/mg* at the same concentration. The ferric reducing

antioxidant activity of the tested fractions of *F. racemosa* crude extract evaluated by FRAP method could be ranked in following order; F19>F15>10>14 > 17>10>11>8>4>6>2>9>3>5>12>16>7>13>16>18 (Figure 2.9c). During the finding of ferric reducing power of 23 fractions of *C. fistula* crude extract, the maximum reducing power was shown by F20(3.1±1.24) and F 22(3.0±1.78) *µg AAE/mg* at a concentration 600 *µg/ml* lies in the range of the reducing power of ascorbic acid (3.9±1.45) *µg AAE/mg.* FRAP of the 23 fractions of *C. fistula* ranked in this order F20>F22>F21>F23>F19,17>F18>F16>F14,11,10,4>F9>F12>F15,7>F5>2,8>F1 (Figure 2.10c). In the case of *S. cumini* eluted 19 fractions maximum ferric reducing power was shown by fraction F19 (3.7±2.5) followed by F16 (3.4±3.21) *µg AAE/mg* at concentration 600 *µg/ml.* the standard ascorbic acid also showed a similar range of ferric reducing power (3.5±1.09) *µg AAE/mg* at same concentration. *S. cumini* all fractions ferric reducing power in sequence is ranked as follow; F19>F16>F17>F15>F18>F13, 9>F11. F10, F8, F6>F12>F2>F3>F1(Figure 3.11c). Fractions having maximum reducing power were eluted with polar solvent.

Total antioxidant capacity estimation (TAC)

By using the phosphomolybdate technique, which is based on the reduction of Mo (VI) to Mo, the total antioxidant capacity was calculated (V). The development of a green phosphomolybdenum (V) indicates the antioxidant sample. Among the tested fractions of *F. racemosa* crude extract, F-15 exhibited maximum total antioxidant capacity (45.41±1.55) *µg/ml* followed by F-19 (38.23±1.33) *µg/ml*, almost equivalent to the standard ascorbic acid total antioxidant capacity (59.32±1.47) (Figure 3.9d).

Among the isolated 23 fractions of *C. fistula* crude extract, the maximum total antioxidant capacity was shown by F-22 (43.1±2.41) and F20(41.17±1.65) *µg AAE/mg* lies in the range of total antioxidant capacity of standard ascorbic acid (59.32±1.06) *µg AAE/mg.* The lowest total antioxidant capacity was shown by F-19 (21.51±2.57) *µg AAE/mg* (Figure 3.10d).

In current study during fractionation total of 19 fractions eluted from *S. cumini* crude extract. Among that all fractions the best total antioxidant power was shown by F-19 (37.13±2.56) and F-16(33.61±1.72) *µg AAE/mg.* This indicate that these fractions possess high antioxidant power because their TAC is in close range to the antioxidant power of standard ascorbic acid (45.32±3.01). While the F-1 (18.97±2.67) and

F10(20.15±2.56) *µg AAE/mg* showed a very weak total antioxidant power (Figure 3.11d).

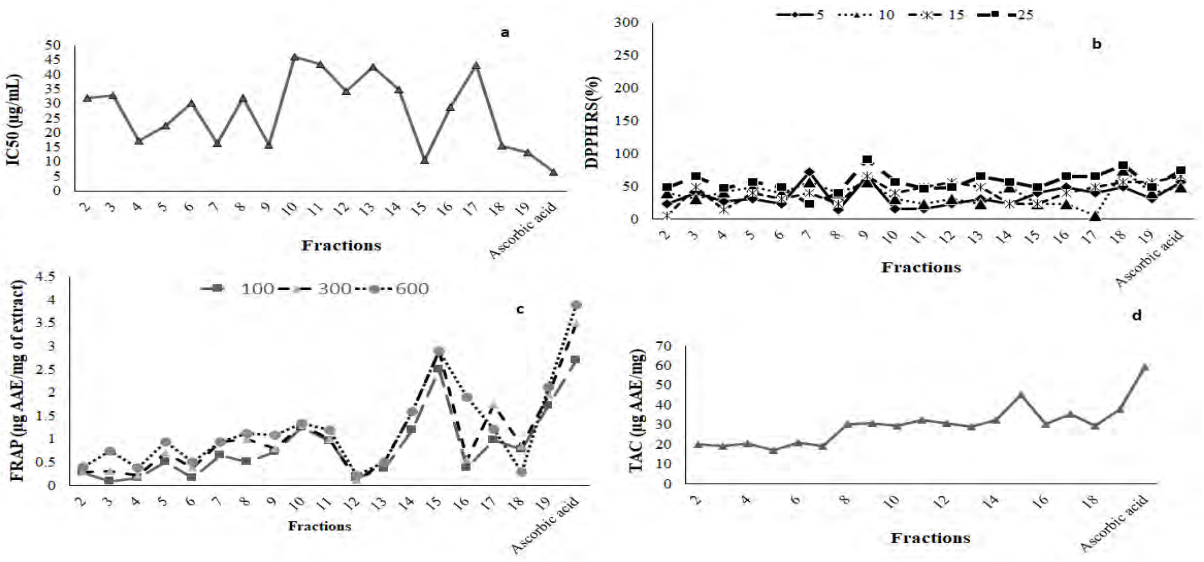


Figure 3.9: (a) Plot of IC50 values (*µ*g/mL) of isolated fractions of *F. racemosa* extract along with standard. **(b)** DPPH radical scavenging percentage of fractions. **(c)** Ferric reducing antioxidant power of fractions. **(d)** Total antioxidant capacity of isolated fractions.

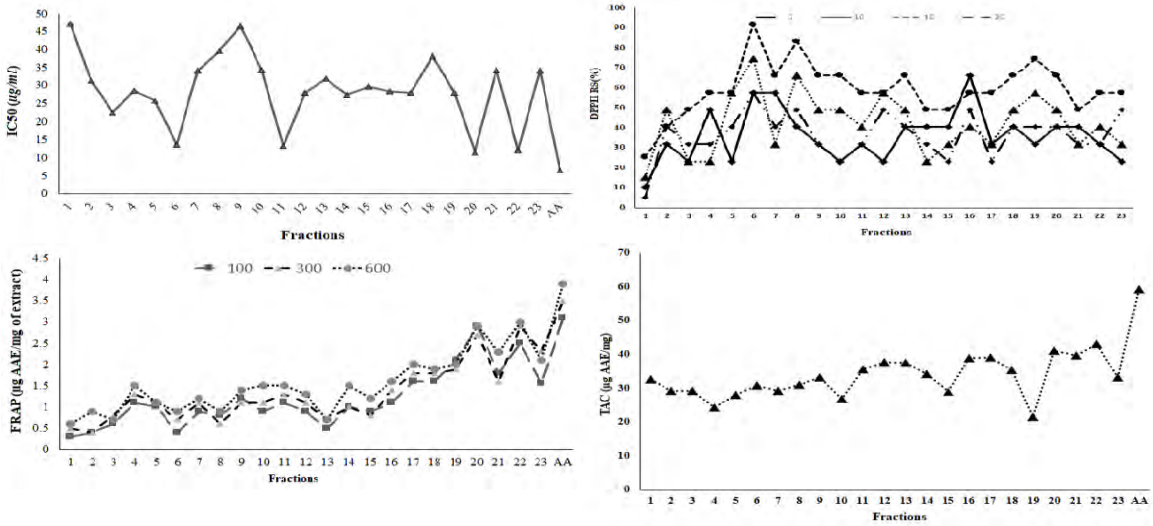


Figure 3.10: (a) Plot of IC50 values (*µ*g/mL) of isolated fractions of *C. fistula* extract along with standard. **(b)** DPPH radical scavenging percentage of fractions. **(c)** Ferric reducing antioxidant power of fractions. **(d)** Total antioxidant capacity of isolated fractions.

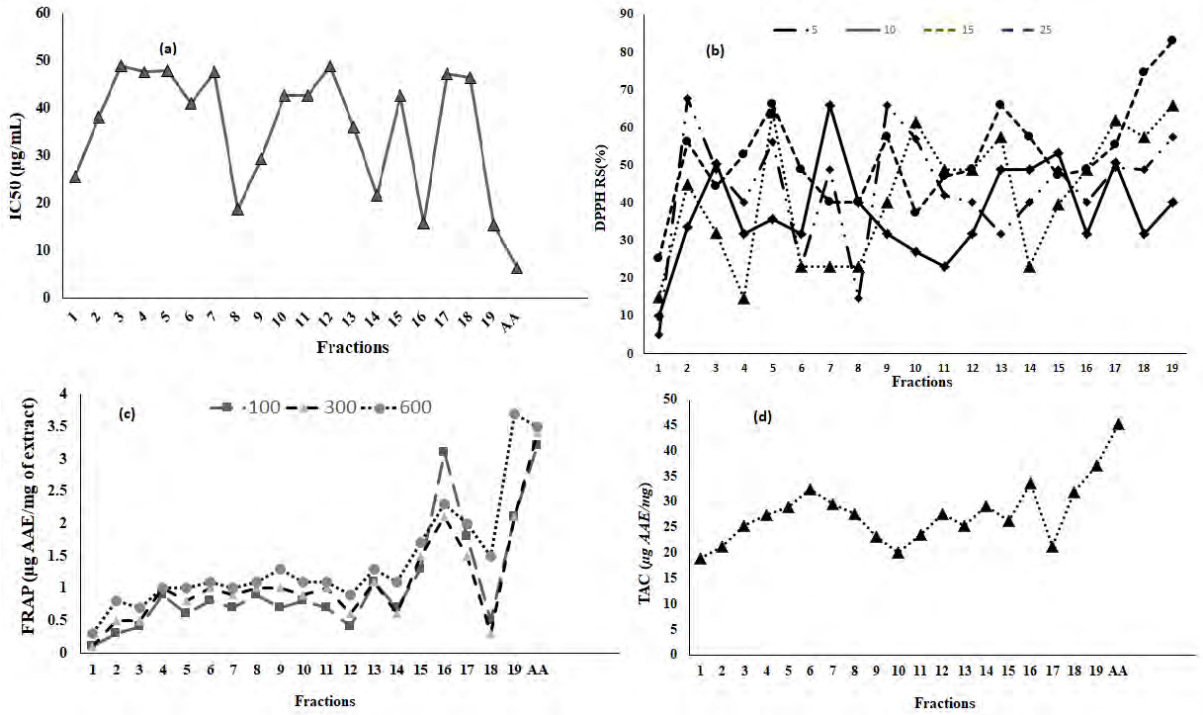


Figure 3.11: (a) Plot of IC50 values (μg/mL) of isolated fractions of *S. cumini* extract along with standard. **(b)** DPPH radical scavenging percentage of fractions. **(c)** Ferric reducing antioxidant power of fractions. **(d)** Total antioxidant capacity of isolated fractions.

Correlation coefficient

The correlation analysis among TPC, TFC and different antioxidant assays was conducted. There is a significant correlation between TFC and DPPH ($R^{2=}.498^{*}$). Similarly, correlation of TPC ($R^{2=}.583^{*}$), TFC ($R^{2=}.474^{*}$) was significant with TAC. A similar trend of significance was observed in TPC ($R^{2=}.556^{*}$) and TFC ($R^{2=}.517^{*}$) correlation with FRAP. While correlation between TAC and FRAP was found to be highly significant ($R^{2}=.782^{**}$). However, correlation between DPPH and TPC was non-significant ($R^{2}=0.256$) (Table 2.4).

***In vitro* anticoccidial assays**

In coccidiosis suspected chick s' gut, on the basis of oocysts morphology, size and site of infection, different species of Eimeria were identified i.e., *E.necatrix*, *E.mitis* and *E.tenella*.

Table 3.4: Correlation between antioxidant activities, total phenolic and flavonoids contents

Parameters	Pearson Correlation	TP	TF	DPPH	TAC	FRAP
TP	Pearson Correlation	-	-	-	-	-
	Sig. (2-tailed)	-	-	-	-	-
	N	18	-	-	-	-
TF	Pearson Correlation R^2	0.355	1	-	-	-
	Sig. (2-tailed)	0.148	NA	-	-	-
	N	18	18	-	-	-
DPPH (IC50)	Pearson Correlation R^2	0.256	.498*	1	-	-
	Sig. (2-tailed)	0.305	0.036	NA	-	-
	N	18	18	18	-	-
TAC	Pearson Correlation R^2	0.583*	.474*	0.054	1	-
	Sig. (2-tailed)	0.011	0.047	0.831	NA	-
	N	18	18	18	18	-
FRAP	Pearson Correlation R^2	.556*	.517*	0.225	.782**	1
	Sig. (2-tailed)	0.017	0.028	0.37	0	NA
	N	18	18	18	18	18

Oocysts sporulation inhibition assay

The sporulation inhibition activities of the isolated fractions of selected plants i.e., *F. racemosa*, *C. fistula* and *S. cumini* against *Eimeria* species oocysts were evaluated. Among different concentrations of each fraction (n=18) of *F. racemosa i.e*., 2.5, 5, 10, 20 and 30*mg/ml*, the highest sporulation inhibition efficacy (67.11±2.18%) was observed by fraction 19 at a concentration of 30*mg/ml* against *E. tenella*. The lowest efficacy (3.31±2.37%) was shown by fraction 7 at concentration 2.5*mg/ml* against *E. mitis* (Figure 3.12). In isolated fractions (n=23) of *C. fistula* crude extract, the maximum sporulation inhibition efficacy (69.11±2.18) and (69.56±1.14)% was shown by F-22 at concentration 30*mg/ml* against *E. tenella* and *E. necatrix* respectively. While the lowest efficacy (4.56±2.05%) was shown by F-4 at concentration 2.5*mg/ml* against *E. mitis* (Figure 3.13). Among the eluted fractions (n=19) *of S. cumini* crude extract F-19 inhibit maximum oocyst sporulation of *E. necatrix* at concentration 30mg/ml showing sporulation inhibition of (76.56±1.14%). While the least oocysts sporulation inhibition was recorded in F9 at concentration 2.5mg/ml against *E.necatrix* having sporulation inhibition of (2.03±2.38%) (Figure 3.14).

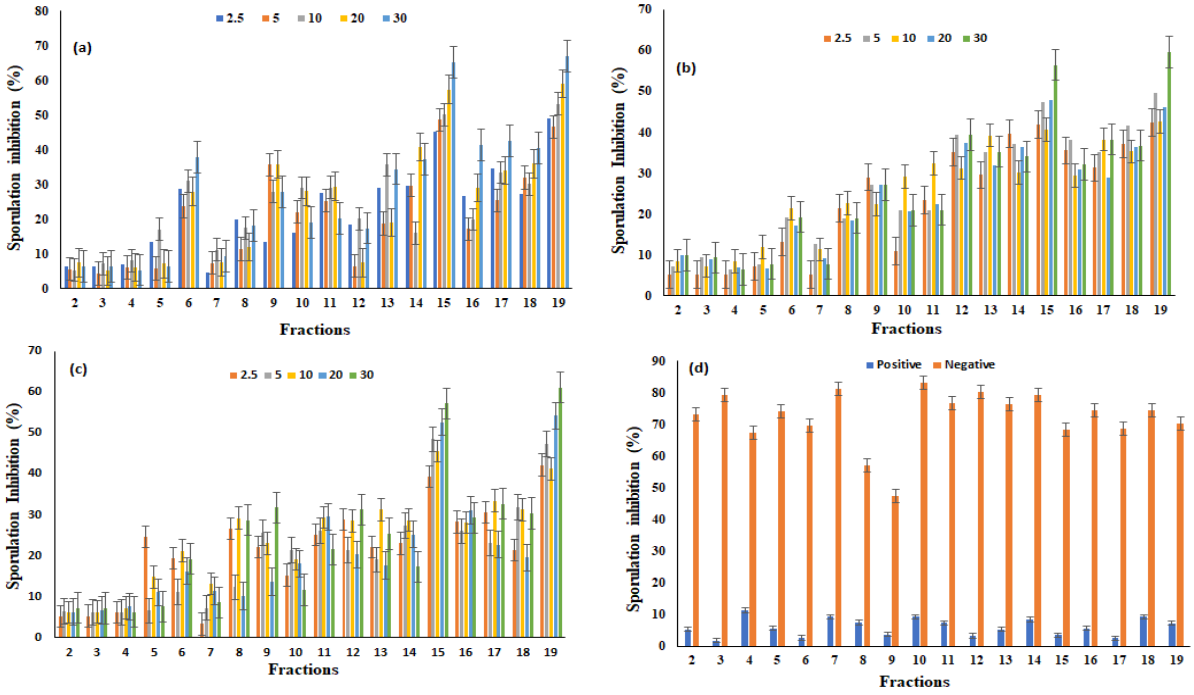


Figure 3.12: *F. racemosa* fractions different concentrations effect on sporulation inhibition (%) of **(a):** *E. tenella,* (**b):** *E. necatrix,* **(c):** *E. mitis* oocysts. **(d)**: Control groups

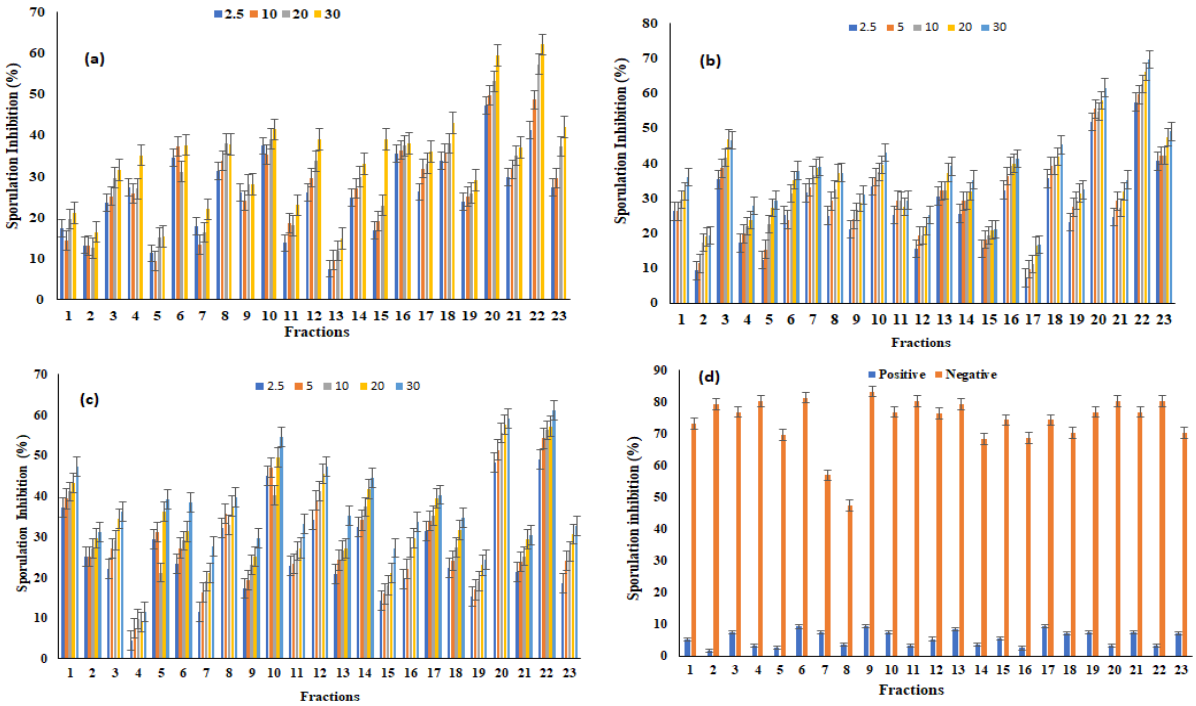


Figure 3.13: *C. fistula* fractions different concentrations effect on sporulation inhibition (%) of **(a):** *E. tenella,* (**b):** *E. necatrix,* **(c):** *E. mitis* oocysts. **(d)**: Control groups

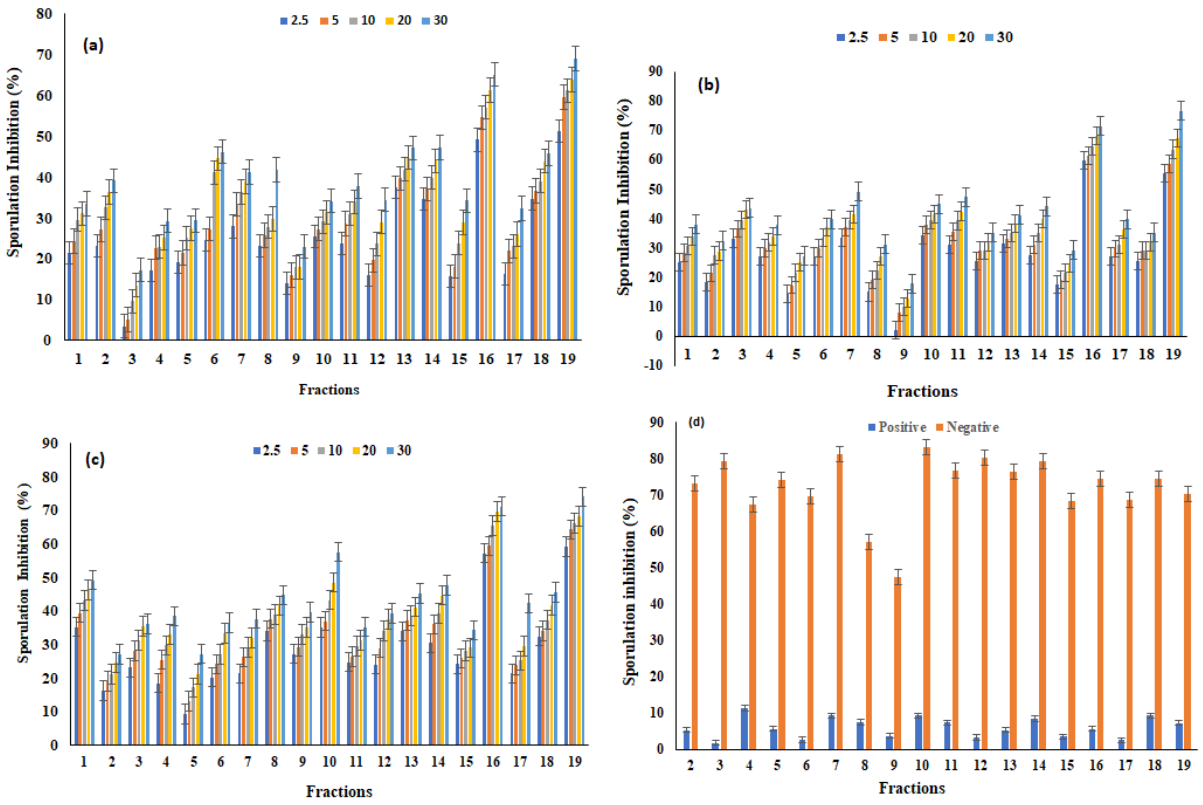


Figure 3.14: *S. cumini* fractions different concentrations effect on sporulation inhibition (%) of **(a):** *E. tenella,* **(b):** *E. necatrix,* **(c):** *E. mitis* oocysts. **(d)**: Control groups

Sporozoites viability inhibition assay

Among different fractions of *F. racemosa*, highest sporozoites viability inhibition (67.14±2.11) was observed in fraction 15 at concentration 30mg/ml against *E. necatrix*. While the lowest sporozoites viability inhibition (2.57±0.00%) was showed by fraction 7 at a concentration of 2.5*mg/ml* against *E. mitis* (Figure 3.15). Among the total eluted fractions (n=23) of *C. fistula* extract the highest sporozoites viability inhibition (71.56±1.23%) was shown by F-20 against *E. mitis* at concentration 30*mg/ml* while the lowest sporozoites viability inhibition (5.25±1.56%) was shown by F-8 at a concentration of 2.5*mg/ml* against *E. necatrix* (Figure 3.16).

In total eluted fractions (n=19) of *S. cumini* extract the highest sporozoites viability inhibition percentage (72.56±1.23%) was shown by F16 at 30mg/ml concentration against *E. mitis.* While the lowest inhibition of sporozoites viability (3.38±1.59%) was observed in F11 at lowest concentration of 2.5*mg/ml* against *E. necatrix* (Figure 3.17).

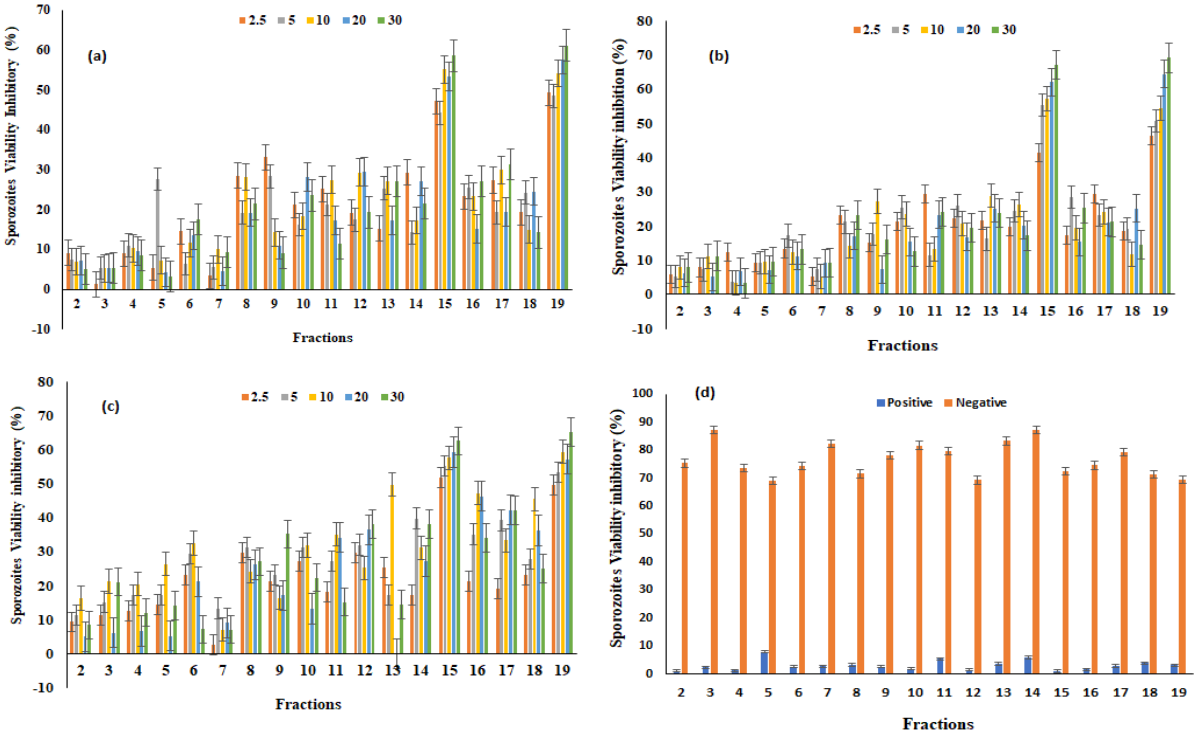


Figure 3.15: *F. racemosa* fractions different concentrations effect on sporozoites viability inhibition (%) of **(a):** *E. tenella,* (**b):** *E. necatrix,* **(c):** *E. mitis* oocysts. **(d)**: Control group

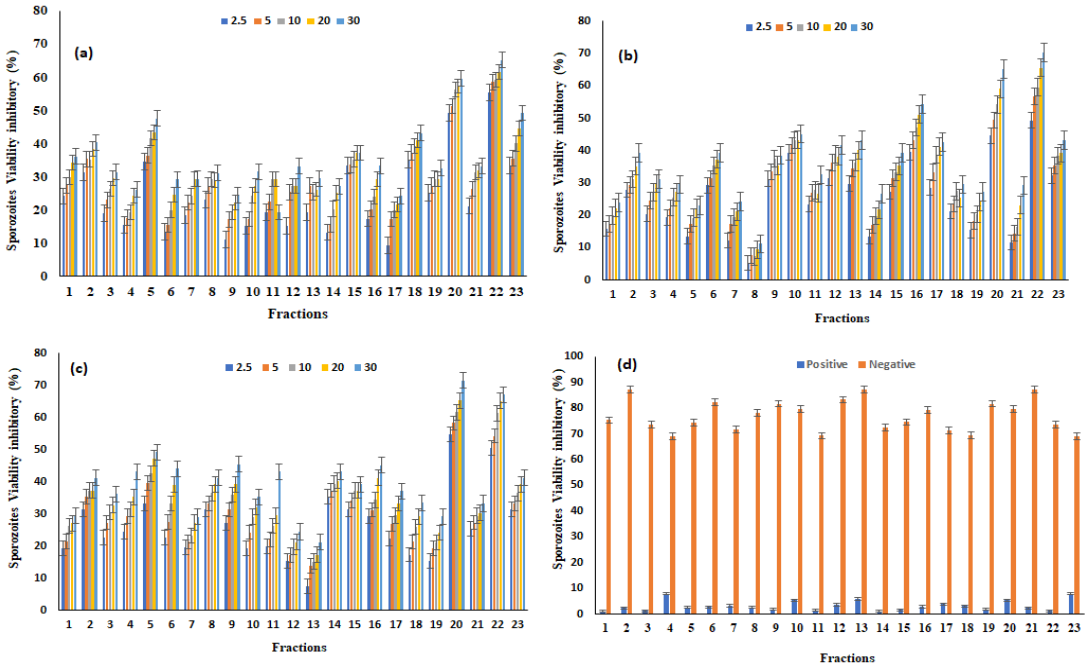


Figure 3.16: *C. fistula* fractions different concentrations effect on sporozoites viability inhibition (%) of **(a):** *E. tenella,* (**b):** *E. necatrix,* **(c):** *E. mitis* oocysts. **(d)**: Control groups

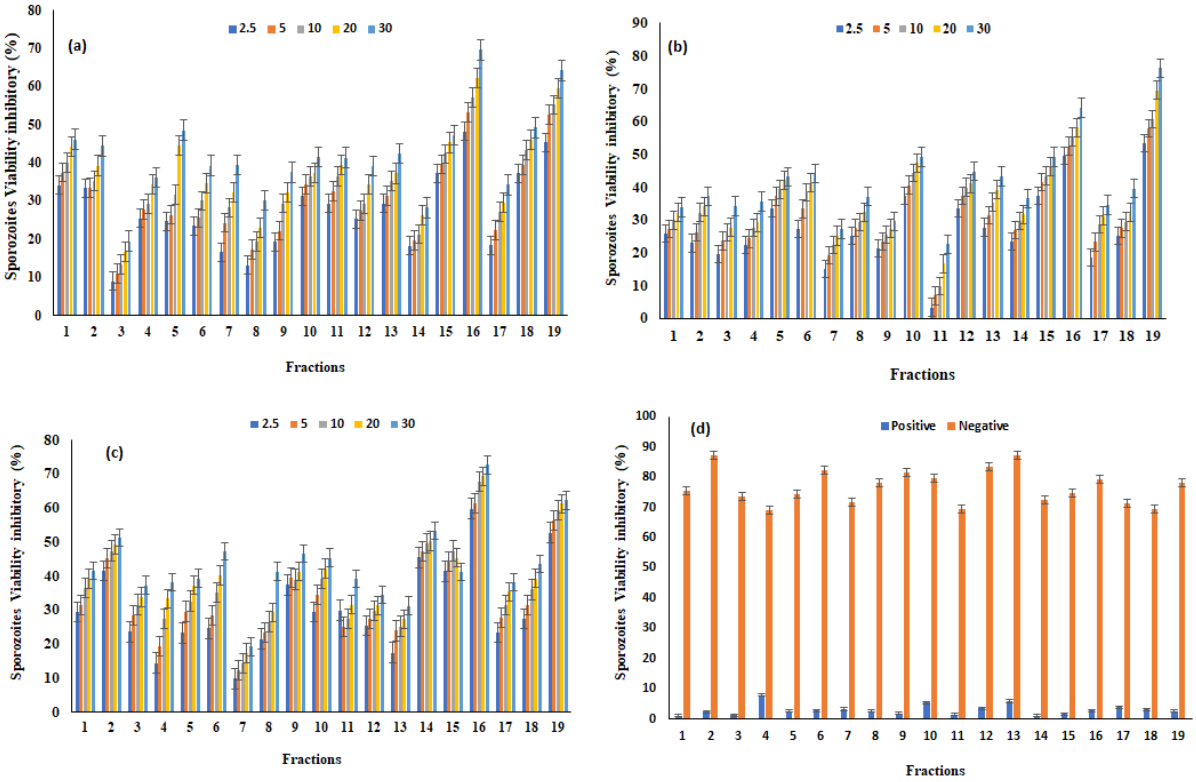


Figure 3.17: *S. cumini* fractions different concentrations effect on sporozoites viability inhibition (%) of **(a):** *E. tenella,* (**b):** *E. necatrix,* **(c):** *E. mitis* oocysts. **(d)**: Control groups

DISCUSSION

According to our knowledge the present study is the first attempt to fractionate the crude methanolic extracts of selected plants, followed by an assessment of the anticoccidial and antioxidant properties of the eluted fractions. During column chromatography for the fractionation of crude extract of selected plants elution started with a non-polar mobile phase and ended with the polar combination. Because plant extracts include a range of polar chemical compounds. By using a mobile phase with different polarities, the various compounds may be separated according to their polarity. Similar to this, Shabrina *et al*., (2018) followed the pattern of increasing polarity in mobile phase isolating *Garcinia Fruticosa lauterb* extract fractions, they followed the trend of increasing polarity in mobile phases during column chromatography. The active fractions of chosen plant crude extracts were subjected to preliminary phytochemical screening using various methods, which confirmed the presence of several secondary metabolites including proteins, carbohydrates, saponin, phenols, flavonoids, terpenoids, and alkaloids. The tannins and phenolic compounds possess antibacterial activities.

The *in vitro* antioxidant properties of the eluted fractions have been assessed in this work using a variety of methodologies to enable quick compound screening. It is well known that an imbalance between the production of ROS and their neutralization causes an excessive amount of oxidative stress, which aggravates illness. Several *in vitro* techniques have been utilized to assess the antioxidant and free radical scavenging capabilities of the majority of naturally occurring compounds, which have been shown to have antioxidant properties (Mujeeb *et al*., 2017). According to our findings, the majority of significant phytochemicals are found in nearly all fractions, but particularly in the bioactive fractions. The biological actions of secondary metabolites of plants are frequently different and distinctive (Appapalam and Panchamoorthy, 2017). Additionally, secondary metabolites in the fractions, including polyphenols, phenolic acids, flavonoids, flavonols, diterpenes, tannins, phytosterols, fatty acid esters, phenylpropanoids, alkaloids, and glycosides, are bioactive compounds classes and due to high antioxidant properties that make them highly significant in medicinal chemistry and natural product research (Maestri *et al*., 2006). The plants antioxidant capacity is correlated with their phenolic content, and phenolics themselves have a high range of

biological functions (Bag *et al*., 2013). Crozier *et al*., (2008) reported that the saponins from plants had antibacterial and anti-inflammatory properties. The tannins were shown to have antimicrobial, antimutagenic, anticarcinogenic, antidiarrheal, and antiseptic activities in earlier investigations (Graça *et al*., 2016). Bagyalakshmi *et al*., (2019) proposed that *F. racemosa* extract contains a wide range of phytochemicals. The results of the current study are consistent with the results of Khan *et al*., (2017) who suggested that phyto-constitutional investigation of *C. fistula* based on chemical testing revealed the presence of flavonoids, tannin, glycoside, saponin, and alkaloid in both methanolic and aqueous extract, respectively. However, the absence of iodine and traces of glycoside in fresh juice and aqueous extract provided proof that the extracting solvent had no impact on the chemical makeup of the plant.

In preliminary phytochemical investigations the eluted fractions of methanolic extract of leaves of *S. cumini* showed the presence of flavonoids, terpenoid, steroids, alkaloids, glycosides, phenols, saponins, cardiac glycosides, and tannins (Gowri and Vasantha., 2010). Significant bioactive components which are found in leaves of *S. cumini* that make the plant a potential antioxidant and anti-diabetic. The research revealed that the majority of phytochemicals, such as alkaloids, flavonoids, saponins, tannins, glycosides, phenols, proteins, triterpenoids, steroids, fixed oils, and lipids, were present in both the ethanolic and methanolic extracts (Ramos and Bandiola., 2017).

Polyphenols was present in each of the leaf and stem bark extracts/fractions. The phenol contents found for each sample and the DPPH findings obtained are consistent. Plant polyphenols are produced from the amino acid phenylalanine or its precursor shikmic acid. As a result of their excellent structural chemistry for free radical scavenging activities and *in vitro* antioxidant efficacy, vitamins E and C are significant dietary antioxidants (Ribeiro *et al*., 2008). Our research showed that certain plant fractions from particular species have high phenolic and flavonoid content levels, which are the primary scavengers of free radicals in oxidation pathways (Das *et al*. 2014).

The overall phenolic content in the current study, as found in the active fractions of chosen plants, varies from (53.27 to 125.57) gGAE /mg, whereas the range for flavonoids is (67.21–125.57) gQE /mg. These total phenolic and flavonoid content levels match the range of ascorbic acid, the control agent used in the current study. Bachaya *et al*., (2015) proposed that various medicinal plants flavonoids and phenolic

compounds have been utilized to treat a wide range of illnesses. The two major phytochemical compounds from plants that have antioxidant capabilities are phenolics and flavonoids (Andreu *et al*., 2018). The concentration of the phenolic constituents was associated with their antioxidant capability (Velioglu *et al*., 1998). Phenols have potent anti-inflammatory, anti-mutagenic, and anticancer effects (Ahmad and Mukhtar, 1999). In foods like vegetables, nuts, fruits, and beverages like red wine, tea, and coffee, flavonoids are present that belong to one of the most important class of chemicals (Hertog *et al*., 1993).

The DPPH radical scavenging model is often used to evaluate the antioxidant activity of natural substances and plant extracts. The level of discoloration reveals the scavenging activity of the antioxidant extract, which is caused by its ability to donate hydrogen (Barreira *et al*., 2008). The results of the current study revealed that all of the fractions of the selected plants have the ability to scavenge free radicals and the activity of DPPH radical scavengers is correlated with dosage. IC_{50} values of DPPH radical scavenging of the active fractions of selected plants range 10.57-15.78 *µg/ml* has been reported. These values are very close to the IC_{50} values range of standard ascorbic acid (6.47±2.78) *µg/ml* in this study. Similarly, in a study conducted by Das *et al*., (2014) reported an IC_{50} value (8.78 *µg/ml*) of *Crescentia cujete* leaves and stem extract. In contrast study conducted by Shabrina *et al*., (2018) proposed that out of 14 fractions two fractions showed highest DPPH radical scavenging activity eluted with hexane and ethyl acetate solvents indicating that compounds eluted were non polar. By measuring the 700 nm Prussian blue production, it was possible to determine that the active fractions of specific plants include reductants (antioxidants), which cause the Fe3+/ferricyanide complex to be reduced to the ferrous form (Das *et al*., 2014). Previous research has demonstrated that the phenolic components are crucial to the extracts' reducing power (Von *et al*., 1997).

The presence of reductants, which serve as antioxidants by donating an atom of hydrogen and breaking the chain of free radicals, is typically linked to the reducing ability of extracts. The reducing power of each of the chosen plants active components indicates that it is most likely to make a major contribution to the total anti-oxidant effect. However, it has been established that plant extracts antioxidant activity works through a variety of mechanisms, including the binding of catalysts for heavy metal

ions, the dissolution of peroxides, the prevention of chain initiation, the reductive capacity of metals, and the scavenging of free radicals (Jan *et al*., 2013). Selected plants active fractions reducing power increased with increasing concentration of extract. However ascorbic acid has a considerably maximum reducing power than present study experimental fractions. Similarly, Jan *et al*., (2013) also reported the same trend of increase in reducing power with increasing in their concentration.

In contrast a study conducted by Feghhi-Najafabadi *et al*., (2019) observed hexane extract containing non polar compounds exhibited maximum reducing power. The development of a green phosphate/molybdenum (V) complex at an acidic pH and subsequent reduction of molybdenum (VI) to molybdenum (V) by extracts are the basis for the overall antioxidant capacity. High values for absorbance meant the sample had a lot of antioxidant activity. The difference in the antioxidant content of extracts and fractions may be explained by variations in the quantity and type of antioxidant chemicals already present in them, such as carotenoids, phenol, and ascorbic acid (Jana *et al*., 2012). This test is based on the development of a green phosphate/MoV complex, which is detected by a spectrophotometer, when phosphor molybdate ion is reduced in the presence of an antioxidant. It is evident from a coefficient correlation analysis of the TPC, TFC, and antioxidant activities of the eluted fractions that there is a strong relationship between these activities. Numerous studies have been published that demonstrate the association between TPC, TFC, and other antioxidant activities (Manzoor *et al*., 2012).

According to Cedric *et al*., (2018), *P. guajava* extracts inhibit growth and development of oocysts at maximum concentration of 30mg/ml. The results of the current study suggest that cell membrane disintegration is the exact mechanism of action of certain plants, despite the fact that this is unclear. This may be due to the presence of phenolic compounds with low molecular weight and a hydrophobic character (Jitviriyanon *et al*., 2016). Another potential mechanism might result from interfering with the physiological process of sporulation, such as a halt in oxygen consumption and the inactivation of crucial sporulation enzymes (Desalegn *et al*., 2020). Results of study conducted by Abbas *et al.,* (2019) found that *T. ammi* extract had a dose-dependent influence on the no of *Eimeria* oocysts that were damaged and

sporulated. Additionally, *T. ammi* extract negatively impacted the size, shape, and number of sporocysts in oocysts.

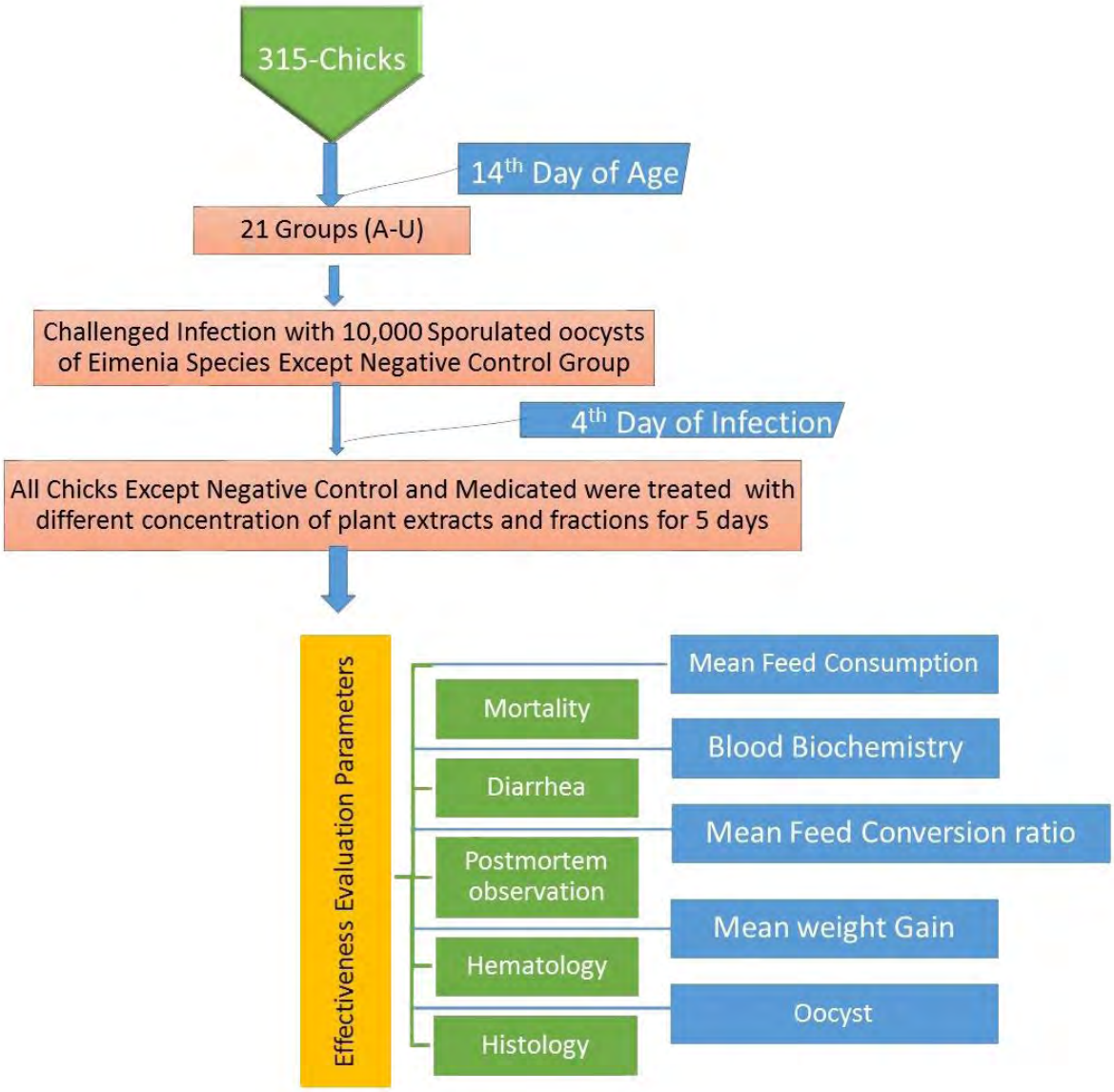
Conclusion

According to the present findings, *F. racemosa, C. fistula,* and *S. cumini* crude leaf extract bioactive fractions possess a variety of phytochemical components and have potential *in vitro* anticoccidial effects. These findings may contribute to the development of a potent substitute for the current anticoccidial medications. More investigation is required in order to identify and isolate the pure bioactive compounds that may be used in the synthesis of reasonably priced natural antioccidial drugs.

Chapter 4

Title: *In Vivo* anticoccidial activity of experimental plants methanolic crude extracts and their anticoccidial fractions

Graphical Abstract



ABSTRACT

A protozoan parasite of the genus *Eimeria* cause coccidiosis. This is an avian infection causing an economic loss of $ 3 billion per year globally in the poultry industry. Although several anticoccidial drugs are in use, which have side effects and are cost-effective, there is a need for more anticoccidial agents. Thus, the anticoccidial activity of different concentrations of crude extracts of *F.racemosa*, *C.fistula*, *S.cumini* and their fractions was tested against mixed *Eimeria* species in broiler chicks (n = 315) in comparison with anticoccidial amprolium. On day 14th, the experimental birds were allotted 21 groups designated as A-U. Total of 5 chicks per group and each group was in triplicate. The chicks were orally infected with 7000–10,000 sporulated oocysts of mixed *Eimeria* species in all experimental groups, with the exception of negative control group T. All groups, with the exception of negative control group T and medicated group S, were administered with different supplements of plant extracts and their fractions orally at a dose of 1 ml/chick daily for 5 days started on day 4 post inoculation (PI), as the first sign of infection appeared. Medicated group S was given amprolium (1.25*mg/m*l) in feed. The effectiveness of experimental doses was evaluated for 36 days based on the average weight increase, feed consumption, feed conversion ratio, oocyst shedding, diarrhea, mortality, blood biochemistry, hematology, histopathology and etc. The acute toxicity of extracts was investigated by using 50one-day old broiler chicks. A total of 10 groups with 5 chicks in each. A maximum oral dose of 2,000 mg kg-1 b.wt of different extracts of selected plants and their anticoccidial fractions were given to all groups, with the exception of the control group. Over the period of 14 days, all the chicks were monitored for any clinical symptoms of toxicity. Drug resistance of *Eimeria* species was assessed by using anticoccidial sensitivity test (AST), anticoccidial index (ACI), and percent optimal anticoccidial activity (POAA) .Among all experimental groups, maximum weight gain was recorded on the 5th week in group N (447±4.04) and M (444±3.05) gm treated with (20%) and (10%) of F-22 of *C. fistula* respectively, which was in a similar range to the weight gain of the group treated with amprolium (443±3.07) gm and the uninfected non-medicated control group (456±2.51) gm. Maximum mean feed consumption was recorded at 5th week of age in groups N (761±0.57) gm and M (759±0.57) gm treated with (20%) and (10%) of F-22 of *C. fistula* respectively, which was higher than the mean feed

consumption of groups treated with amprolium (725±1.52)gm and in the range of uninfected non-medicated control group S(762±1.15) gm. The best FCR results were shown by group N. Among all experimental groups, the maximum reduction in oocyst shedding was recorded in group M treated with 10% of F-22 of *C. fistula*. No mortality and diarrhea were observed in the best treated groups. Normal histology, serum biochemistry, and hematology were observed in groups treated with different concentrations of anticoccidial fractions of selected plants. In the acute toxicity study, till the 14^{th} day, there was no mortality. While *F. racemosa* and *C. fistula* crude extracts treated chicks showed tremors, lethargy and etc. Based on a combined analysis of the three indices used in the current study, an overall drug sensitivity assessment was carried out and it was concluded that group A and S showed strong resistance, B and Q showed moderate resistance, groups E-J, P showed slight resistance, and the rest of the groups showed sensitivity to their respective treatments. In summary, the studied herbal complexes' concentration-dependent anticoccidial activity points to their potential use as an alternative anticoccidial agent for the control of *Eimeria* species. It is recommended that large-scale investigations be conducted to identify and isolate bioactive compounds from the investigated plants' anticoccidial fractions.

INTRODUCTION

The most common parasitic disease is considered to be poultry coccidiosis in commercial poultry, having a devastating $3.2 billion economic impact on the poultry sector globally each year (Ott et al., 2018). According to some earlier estimates, the global chicken sector suffers coccidiosis-related losses of USD 3 billion every year (Ramadan et al). Coccidiosis is an illness of the small intestine caused by intracellular protozoan parasite Eimeria, which results in localized lesions and lowers the performance of cattle and poultry by reducing their ability to absorb nutrients (Leung et al., 2019). Chicks are susceptible to seven kinds of coccidian that are significant pathologically. Many gastro intestinal bacterial poultry illnesses are predisposing factors, and the digestive tracts mega lesions are one of them. Diarrhea, slower weight growth, and weakness are all consequences of parasite presence in the colon, which causes epithelial cells to shed and absorption to be disrupted (Lin *et al*., 2020). Anticoccidial drugs have been used as feed additives or in drinking water for the prevention and treatment of avian coccidiosis (Arczewska-Wlosek *et al*., 2013). However, due to their regular usage, *Eimeria* strains have emerged in different regions of the world that are resistant to widely marketed anticoccidial medications (Zhang *et al*., 2020). One of the commonly mentioned substitutes for anticoccidial drugs is vaccination (Khater *et al*., 2020), along with plant extracts and essential oils, antioxidant compounds, probiotics, and prebiotics (Jahan *et al*., 2019).

Researchers are currently looking at a variety of herbs to see whether they could be used as preventative measures and treatments for a variety of infectious diseases (Pangasa *et al*., 2007). The immunological response in birds has been found to be triggered by a variety of plants, either in whole or in particular sections, enabling superior immunogenic performance against invasive infections. Literature has also shown that herbal medicines are more widely accepted than synthetic pharmaceuticals since they are less expensive and have less negative effects (Akhtar *et al*., 2012). Several therapeutic plants have been found due to their antimicrobial properties *i.e., Cassia fistula, Psidium guajava, Phyllanthus niruri*, *Ocimun basilucum*, *Rosmarinus officinalis*, *Ehretia microphylla*, *Thymus vulgaris* (Shahid *et al*., 2011: Parivuguna, 2008: Hamilton, 2004). Many have protozocidal potential *e.g*., *Suregada*

zanzibariensis, Albizia coriaria, Aspergillus racemosus, Acacia tortilis (Kigondu *et al*., 2009) and many others for controlling parasites.

Some of the medicinally significant plants were chosen, such as the family Moraceae member *Ficus racemosa*, often known as the gural or cluster fig (Joy *et al*., 2001). This plant is a well-known medicinal plant in India and has been used for many years in Ayurveda, the country's ancient medical system, to cure a range of diseases and problems, including hemorrhoids, liver issues, diarrhea, diabetes respiratory and urinary illnesses. Pharmacological research on *F. racemosa* focuses on its antibacterial, anti-inflammatory, antitussive, hepatoprotective and antidiabetic properties. Numerous phytochemical components from different sections of *F. racemosa* have been discovered and extracted (Ahmed *et al*., 2010).

Cassia fistula locally known as golden shower is a member of the leguminose family native to Sri Lanka, the Amazon, and India, the plant is now widely planted as an ornamental tree (Elysha *et al*., 2015). In addition to being used as a medication to treat rheumatism, hematemesis, pruritus, leucoderma, and diabetes, *C. fistula* has beneficial use against various disorders including skin infections, liver problems, and tuberculous glands. *C. fistula* has been found to have significant antimicrobial properties on several bacteria. This plant is utilized as a broad-spectrum antibacterial agent to treat several infectious disorders due to these characteristics (Duraipandiyan and Ignacimuthu., 2007; Panda *et al*., 2011). *Syzygium cumini* locally known as Jaman, a huge tree belonging to the Myrtaceae family that is also known as Indian blackberry, is found in the Upper Gangetic Plains, Bihar, Orissa, West Bengal, Deccan, Konkan area, and several districts in South India. The medicinal herb S. cumini is used to treat number of illnesses. Containing a variety of chemical components that form the basis for pharmacological action. There have been several reports of this plant's pharmacological characteristics, including antiplaque, antimicrobial, anti histaminic, anti pyretic, gastro intestinal, antioxidant, anti-inflammatory and etc (Jagetia *et al*., 2017). Because of this, the current study assessed the *in vivo* anticoccidial efficacy of several fractions of methanolic extract of experimental plants on chicks infected with mixed species of *Eimeria.*

MATERIALS AND METHODS

Rearing of chicks for experiment

The Quaid-i-Azam University's bioethical committee in Islamabad, Pakistan, authenticated and granted approval for the experimental design used in the study. From a local chicken hatchery in Islamabad, Pakistan, day-old broiler chicks were purchased. In a coccidian-free environment, experimental chicks were reared in an animal house. Prior to housing the chicks, the rooms and cages were cleaned with detergent and disinfectant (Wajiha *et al*., 2018). Each chick received coccidiostat free feed except the control group till the end of the experiment. For first two weeks (1-14 day of age) chicks were given a broiler starter ration, then over the subsequent developing stages (15-36 day of age) broiler finisher ration was given (Herrero-Encinas *et al*., 2020) (Table 4.1). Feed and water were provided ad libitum. Vaccination against infectious bronchitis (H120) was given to chicks through subcutaneous injection on day 5. On day 5, the Lasota strain of the Newcastle Disease vaccination was given. Vaccination against infectious bursal disease (oil-based) was given intravenously on days 8 and 16 (Abbas *et al*., 2017).

Table 4.1: Composition of broiler starter and finisher ration

S.No	Ingredients	Starter(%)	Finisher (%)
1	Corn	48	50
2	Soyabean meal	15	12
3	Rice	10	12
4	Fish meal	3	3
5	CGM 60%	2	3
6	Molasses	3	4
7	DCP	1	1
8	Premix	0.68	0.68
9	DL-Methionine	0.12	0.12
10	L-Lysine	0.2	0.2
11	Total	100	100
Calculated Analysis			
1	Metabolizable energy (Kcal/Kg)	2900	3000
2	Crude proteins (%)	20	18
3	Crude fibers %	3.1	4
4	Calcium %	1.02	0.8
5	Available phosphourous %	0.65	0.4
6	lysine %	1.1	1.1
7	Methionine %	0.5	0.5

During first week, the temperature was kept at 85-90°F, but it was reduced by 5 °F on a weekly basis. Throughout the experiment, light was provided continuously (Zaman et al., 2012).

Determination of maximum tolerated dose by Acute toxicity study

The acute toxicity test was carried out in accordance with guidelines provided by the World Health Organization (WHO) and Organization for Economic Co-operation and Development (OECD). For this investigation, one-day old, 50 commercial broiler chicks were chosen. Each of the chick wings were tagged individually, weighed and placed in cages for 21 days. Five chicks were randomly placed in each of ten groups for the assessment of high dosages of methanolic extracts of selected plants and their bioactive fractions. The control group received distilled water while the other experimental groups were given a single oral dosage of 2,000 mg kg-1 b.wt of various extracts of chosen plants and their selected fractions. Over the course of 14 days, all chicks were observed for clinical symptoms of poisoning, including as tremor, loss of appetite, unsteady walking, painting, eye blinking, lethargy, a lack of coordination, salivation, and death (Hashemi *et al*., 2008).

Experimental design

On 14^{th} day of age all the chicks (n = 315) chicks were divided into 21 groups (A-U). Each group was in triplicate, so a total of 63 groups with 5 chicks per group. Different concentrations of selected plant crude extracts, their bioactive fractions and control groups along with their assigned groups as follows;

Group A: *Ficus racemosa* 10%(W/V)

Group B: *Ficus racemosa* 20% (W/V)

Group C: *Cassia fistula* 10% (W/V)

Group D: *Cassia fistula* 20% (W/V)

Group E: *Syzigium cumini* 10% (W/V)

Group F: *Syzigium cumini* 20% (W/V)

Group G: *Ficus racemosa* Fraction 15 (10%)

Group H: *Ficus racemosa* Fraction 15 (20%)

Group I: *Ficus racemosa* Fraction 19 (10%)

Group J: *Ficus racemosa* Fraction 19 (20%)

Group K: *Cassia fistula* Fraction 20 (10%)

Group L: *Cassia fistula* Fraction 20 (20%)

Group M: *Cassia fistula* Fraction 22 (10%)

Group N: *Cassia fistula* Fraction 22 (20%)

Group O: *Syzigium cumini* Fraction 16 (10%)

Group P: *Syzigium cumini* Fraction 16 (20%)

Group Q: *Syzigium cumini* Fraction 19 (10%)

Group R: *Syzigium cumini* Fraction 19 (20%)

Group S: Medicated (Amprolium 1.25*mg/ml*)

Group T: Negative control

Group U: Positive control

Parasite inoculation and treatment in experimental groups

Prior to the infection, the fecal litter from all groups and subgroups was sampled and inspected for the presence of any coccidian parasite. No group was found positive for oocysts. From processed sporozoites stored at 4°C, potassium dichromate was removed by centrifugation. Sporulated oocysts 7000 to 10,000 of mixed *Eimeria* species were given orally by using dropper to all chicks except experimental groups. On day 4th post inoculation (PI), as the first sign of infection appeared, all groups, with the exception of the negative control and medicated group, received various dietary supplements of plant extracts and their fractions orally at a dose of 1 ml/chick daily for 5 days using a dropper. The medicated group was given amprolium (1.25*mg/ml*) in feed. A period of 5 days was selected for the treatment with different plant extracts, as the oxidative stress induced by the coccidian parasite is approximately 5 days (Naidoo *et*

al., 2008). The effectiveness of selected plants crude extracts and their fractions was evaluated on the basis of following parameters.

Performance Parameters

Determination Of Mean Weight Gain

On the 14th day of age (1st day of challenge infection), chicks in each group were weighed collectively and then reweighed on the 3rd, 4th and 5th weeks by using balance in the morning before feeding. On the 14th day of age the weight of chicks was considered the initial weight. The mean weight of each group was calculated by using the following formula;

MWG= (Mean final weight of all chicks in a group)-[(Mean initial weight of all chicks in that groups)+(Weight of dead chicks)] (Zaman *et al*., 2012).

Feed consumption estimation

A standard quantity of feed was given to each group throughout the experimental period. The amount of feed consumed by each subgroup was calculated as follows; Each morning, the weight of the feed given to each group was measured, and the weight of the feed remaining the next morning was subtracted (Naidoo *et al*., 2008).

Feed conversion ratio determination

The feed conversion ratio (FCR) is the quantity of feed that chickens must consume in order to gain one gramme of body weight. From the 3rd to 5th week of age, FCR was calculated. Due to the fact that the chicks were fed in group, the feed conversion ratio for a specific chick cannot be determined. The following formula was used to determine the feed conversion ratio (FCR) (Ahmad *et al*., 2018).

$$FCR = \frac{Total\ feed\ consumed\ per\ group\ (g)}{Weight\ gain\ of\ live\ chicks + Weight\ gain\ of\ dead\ chicks}$$

Oocysts per gram of feces (OPG)

For microscopicFecal samples from each group were collected from 5th to 10th day post infection for microscopic examination of oocysts (Christaki *et al*., 2004). McMaster's technique was used for counting the oocysts. 3 grams of fecal sample were mixed with 42ml of tap water in a beaker. In case if pellets were present they were

broken and suspension was kept for 12 to14 hours. Suspension was then passed through two folds' muslin cloth.

For calculating the oocysts count per gram of fecal sample following formula was used,

$$X/0.15 \times 45 \times 10 \times 1/3 = X1000$$

Where X= Average oocyst count in 1 chamber, 0.15= Volume under ruled area of McMaster slide, 45= Total volume of suspension, 10= 1/10 dilution factor, 1/3=Correction factor for 1gram of feces (Zaman *et al*., 2012).

Bloody diarrhea

Bloody diarrhea was observed from 4th to 7th day of infection. The extent of bloody diarrhea was assigned as one of five levels, where the zero or (-) level is the normal status, 1 or (+) corresponds to less than 33%, 2 or (++) to corresponds 26–50%, 3 or (+++) to 51–75%, while 4 or (++++) to over 75% bloody feces in total feces (Christaki *et al*., 2004).

Lesion score

Birds were euthanized on 7th and 14th day of infection from each group. After post mortem intestinal tract of each bird was examined and lesion score of 0-4 was assigned to each group (Qaid *et al*., 2020).

Mortality percentage

Mortality percentage was calculated on 36th day of age using the following formula;

$$Mortality\ Percentage = \frac{Total\ No\ of\ dead\ chicks\ in\ group}{Initial\ No\ of\ Chicks\ in\ group} \times 100$$

(El-Sawah *et al*., 2020).

Survival percentage

At 36th day of age survival percentage of each group was calculated as follows;

$$Survival\ Percentage = \frac{No\ of\ live\ chicks\ at\ the\ end\ of\ experiemnt}{Total\ no\ of\ Live\ chicks} \times 100$$

Biochemical analysis

Blood samples (3 ml) were collected from the wing veins of three selected chicks from all groups on the 36th day of age with the help of syringes. The blood samples were collected in vacutainers without anticoagulant and centrifuged at 750 g for 15 minutes at 4°C. The serum was stored in Eppendorf's tubes at –20°C until analyzed (Zaman *et al*., 2012). Albumin, uric acid, creatinine, aspartate aminotransferase(AST),alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined by using commercial kits and biochemical analyzers.

Histopathology

Tissue samples of liver, caecum, and intestine were taken from all groups on the 36th day of the experiment after post mortem. Tissues were fixed with 10% formalin for further processing. Samples were washed with different concentrations (70, 80, 90, 95 and 100%) of ethanol and cleared in xylene for 60-90 minutes. The specimens were embedded in paraffin wax for 120 minutes and ribbons were made after sectioning at 7µ thickness by a microtome machine. A thin layer of albumin was smeared on slides by placing them on a slide warmer. Ribbons on albumin coated slides were placed in xylene, 100, 90, 70% ethanol for 10 minutes. Slides were gently washed with water for 2 minutes. Then stained with hematoxyline for 10 seconds and again washed with running water for 5 minutes. Eosin stain was applied for 1 minute and de-stained with 95% ethanol. Slides were coated with four drops/slide of permount and observed under light microscope (Yazdanabadi *et al*., 2020; Kadhim, 2014).

Organ weight

At 36th day of trial, all remaining chicks from each group were weighed and slaughtered individually. Internal organs i.e., liver, spleen, intestine, bursa, kidney and heart were removed surgically and weighed using weighing balance.

Percent Organ Relative weight= (organ weight (g)/body weight of animal on sacrifice day (g) × 100.

The data was expressed as per cent organ weights ratio relative to the live body weight (Awais *et al*., 2011).

Ileal digesta viscosity analysis

Some chicks were randomly selected from each triplicate of all groups and slaughtered on day 36th for the determination of the viscosity of ileal digesta. The

gastrointestinal tract was dissected aseptically immediately after slaughter, and the intestinal content was exposed. The ileal digesta content was collected from the Merckel's diverticulum to the ileocaecal junction. Uniform weights of the samples (5 g) were taken from each bird using a sensitive scale and diluted to a volume of 400 ml. The ileal contents collected were centrifuged at (2,500 g for 15 min). The supernatant was withdrawn and the viscosity was measured in a Brookfield viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA) (Oke *et al*., 2017; Waldenstedt *et al*., 2000).

Hematological Evaluation.

Blood was collected to evaluate hematological parameters by using a 3 mL sterile syringe and a 23-gauge needle. In order to determine the total erythrocyte and total leucocyte count, differential leucocyte count, hemoglobin concentration, and packed cell volume values, blood samples were taken from the brachial vein at 5, 7 and 9 DPI in each group. For hematological examination, 1 ml of the 3 ml of whole blood drawn from each group was immediately transferred into a 3 ml sterile tube containing the anticoagulant EDTA. The remaining blood samples were left to clot to prepare sera for serum enzyme assay (Samir *et al*., 2010). Hemoglobin (Hb) value in g/dL was estimated as per the methods described by Benzamin (1985). The graduated measurement tube was put into the hemometer after being filled with a tenth of regular hydrochloric acid up to graduation mark 2. The blood sample was mixed, and then pulled up to the 20 point in the pipette. The measuring tube containing the acid was then filled with the blood, and the pipette was washed by being drawn into the solution three times. Within five to ten minutes, the haemoglobin was transformed into acid haematin of a brown color. After 10 minutes one tenth of normal hydrochloric acid was added drop by drop. It was added slowly till the color matched with the standard on either side of the haemometer. The level of the solution in the tube (upper meniscus) was read and hemoglobin value was expressed as *g/dl.*

Packed cell volume

The Hawskey micro hematocrit technique was used to calculate the packed cell volume (PCV) in percentage (Schalm *et al*., 1996). Blood filled the capillary tubes to a height of 3/4 of their length. The tubes were placed in a unique micro haematocrit centrifuge that had a head designed to hold up to 24 capillary tubes and were sealed at one end with clay. The capillary tubes were placed in a circle, with

the open end in the middle and the sealed end facing outward. The micro haematocrit centrifuge, which was appropriately covered, programmed to spin at 12,000rpm for 5 minutes. Micro haematocrit reader was used to read the PCV value in percentage.

Total erythrocyte count

Following Coles instructions, Hayem's diluting fluid was used to compute the total erythrocyte count (TEC) in millions per cubic millimeter (106/mm^3). The red blood cell pipette was filled with blood up to the 0.5 mark on the stem at first. After then, the pipette was filled with dilution fluid until it reached the 101 mark. After two minutes of gentle mixing, the pipette's contents were discarded, and any extra blood was drawn into a hem cytometer by gently brushing the pipette's tip against a glass slide. Out of 25 tiny squares, 5 small squares (4 corners and 1 center) had their RBCs counted in total, and the total was calculated.

Total leukocyte count

According to Benzamin's approach, the total leucocyte count (TLC), measured in thousands per cubic millimeters (103/ mm^3), was determined using the conventional dilution procedure. A blood sample was diluted with fluid up to the mark of 0.5 in a pipette for WBCs, then well mixed by Thomas. By gently stroking the pipette tip on a glass slide, extra blood was expelled, and blood was then charged into the hemocytometer. Each of the hemocytometer's four corner squares' cell counts were recorded. To calculate the blood count of WBCs/mm^3, the total number of WBCs in the four corners was multiplied by 50.

Differential cell count

At 5, 7, and 9 days after infection, a brachial blood smear was performed. Giemsa stain was used to stain the smears. The proportion of each kind of leukocyte present in the stained smear was calculated. Until 100 white cells had been counted, each was noted on a differential cell counter. The various WBC subtypes were represented as percentages.

Complete blood count (CBC)

On the 36^{th} day of the experiment, a CBC was done to evaluate the blood's hematology. Absolute counts of heterophils and lymphocytes as well as the H/L ratio were calculated, along with packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). PLT, lymphocytes, and RDW measure the distribution of red

blood cells (Gotep *et al*., 2016).

Post mortem Observations of Organs

On 36th day of age after slaughtering chicks, their gross organ postmortem observation was done for any change in their morphology, size and *etc* (Sharma *et al*., 2015).

Evaluation of resistance

Drug resistance of *Eimeria* species was evaluated by using the following three indexes (Ojimelukwe *et al*., 2018).

The Anticoccidial Index (ACI)

The anticoccidial index of anticoccidial drugs against *Eimeria* species was calculated by using the following formula:

$$ACI = (Rate\ of\ relative\ Weight\ gain + Survival\ rate)\text{-}(Lesion\ Score + Oocyst\ Value)$$

An ACI value of ≥ 160 indicated sensitivities; a value < 160 indicated resistances (Fei *et al*., 2013).

Oocyst value was calculated as follows:

$$Oocyst\ Value = \frac{OPG\ output\ of\ each\ Group}{OPG\ output\ of\ Positive\ Control\ Group} \times 100$$

The Anticoccidial Sensitivity Test (AST)

$$AST = \frac{Average\ Lesion\ Score\ in\ infected\ unmedicated\ group\text{-}Average\ Lesion\ Score\ in\ medicated\ group}{Average\ lesion\ score\ in\ infected\ unmedicated\ group} \times 100$$

AST ≥ 50% was judged to be sensitive and < 50% was resistance (McDougald *et al*., 1987).

Percent Optimum Anticoccidial Activity (POAA)

$$POAA = \frac{GSR\ in\ medicated\ group - GSR\ in\ infected\ unmedicated\ group}{GSR\ in\ uninfected\ unmedicated\ group - GSR\ in\ infected\ unmedicated\ group} \times 100$$

GSR (Growth and Survival Ratio) was defined as final body weight divided by initial

body weight.

POAA > 50% was judged to be sensitive and ≤50% was resistance (Arabkhazaeli *et al*., 2013). The overall assessment of drug resistance of *Eimeria* isolates was adapted from Lan *et al*., (2017). Briefly, if 3 of 3 indexes showed resistance, our isolate was considered as severely drug resistant (+++). Two of 3 meant moderate drug resistance (++), 1 of 3 meant slight drug resistance (+) and none meant no drug resistance.

RESULTS

Results of *in vivo* trials on evaluation of anticoccidial activity of selected plants and their fractions are given as under. Although all chicks experimentally challenged with *Eimeria* spp. showed clinical signs and symptoms of depression, ruffled feathers, hemorrhagic faeces, weakness, decreased activity, with death of some chicks. From 5th day post inoculation symptoms started, being maximum at 7th and 9th day post infection and then disappearance of bloody diarrhea starts till the end of the first week. The symptoms were mild in supplemented in some groups like (C, D, K, L, M, N, S, T) than other groups (A,B,E ,F, G, H, I, J, O, P, Q, R, U).

Acute toxicity study

Chicks treated with herbal extracts and their fractions at 2000 mg kg^{-1} body weight did not show any immediate behavioral changes. The chicks moved and drank normally immediately after administration. Chicks treated with *F. racemosa* crude extract showed tremor, chicks treated with *C. fistula* crude extract showed lethargy. other groups don't show any symptoms of toxicity. There was no mortality till 14th day after administration (Table: 4.2).

Table 4.2: Toxicity signs observed during 24 hours in chicks received 2000*mg/kg b.w* oral dose

Samples	Toxicity Signs								
	Tremor	In appetance	Unsteady Gait	Panting	Eye Blinking	Lethargy	In Coord-ination	Sali-vation	Death
F.racmosa	P	A	A	A	A	A	A	A	A
C.fistula	A	A	A	A	A	P	A	A	A
S.cumini	A	A	A	A	A	A	A	A	A
FR F-15	A	A	A	A	A	A	A	A	A
FR F-19	A	A	A	A	A	A	A	A	A
CF F-20	A	A	A	A	A	A	A	A	A
CF F-22	A	A	A	A	A	A	A	A	A
SC F-16	A	A	A	A	A	A	A	A	A
SC F-19	A	A	A	A	A	A	A	A	A
Control	A	A	A	A	A	A	A	A	A

P=Presence, A=Absence

Effect of *C. fistula*, *F. racemosa* and *S. cumini* extracts and their anticoccidial active fractions on growth performance of chicks infected with mixed *Eimeria species*

Changes in mean body weight gain (g) of coccidia parasite infected chicks, during and after treatment with 10 and 20% of *F. racemosa*, *C. fistula* and *S. cumini* leaves methanolic crude extracts and their active fractions were investigated between 3rd and 5th week of age. The results showed that body weight gain of uninfected non-medicated control group was higher than all herbal medicated groups and the infected non-medicated control group. There was significant difference of ($P<0.001$) between the body weight gains of groups supplemented with 10, 20% *F. racemosa, C. fistula* and *S. cumini* crude extracts, their bioactive fractions and amprolium (1.25mg/ml) treated groups. Maximum weight gain was recorded on 5th week in group N (447±4.04) and M (444±3.05) gm treated with (20%) and (10%) of F-22 of *C. fistula* respectively which was in similar range to the weight gain of group treated with amprolium (443±3.07) gm and uninfected non-medicated control group (456±2.51) gm. Followed by group L (426±1.33) and group K (423±4.16)gm treated with(20%) and (10%) of F20 of *C. fistula*. Lowest weight gain was shown by group E on 3rd week of experiment treated with 10% *S. cumini* crude extract (224±1.73)gm and group P (226±11.53)gm treated with fraction 16 (20%) of *S. cumini* lies in the range of positive control group U mean weight gain (148±10.81)gm (Table 4.3).

Effect of *C. fistula*, *F. racemosa* and *S. cumini* extracts and their anticoccidial active fractions on mean feed consumption of chicks infected with mixed *Eimeria species*

Maximum mean feed consumption was recorded at 5th week of age in groups N (761±0.57) gm and M (759±0.57) gm treated with (20%) and (10%) of F-22 of *C. fistula* respectively which was higher than the mean feed consumption of group treated with amprolium (725±1.52)gm and in the range of uninfected non-medicated control group (762±1.15) gm. Followed by groups L (715±0.57) and K (713±2.5) gm treated with (20) and (10)% fraction 20 of *C. fistula* respectively. While the least amount of mean feed consumption was recorded on 3rd week in group P treated with (20%) of F16 of *S. cumini* similar to the positive control group U mean feed consumption i.e., (396±1.73)gm (Table 4.4).

Effect of *C. fistula*, *F. racemosa* and *S. cumini* extracts and their anticoccidial active fractions on mean feed conversion ratio of chicks infected with mixed *Eimeria species*

Lowest FCR (1.23±0.20) and (1.33±0.05) was shown by amprolium medicated group S and negative control group T respectively in 3rd week of experiment. From the time of infection to recovery due to lower feed consumption, feed conversion ratio of all infected groups were higher than uninfected non-medicated control group, as result all chicks lost weight. Among experimental treated groups the best results were shown by group N by having lower FCR ratio (1.25±0.01) treated with (20%) of fraction 22 of *C. fistula.* Followed by group D treated with (20%) of crude *C. fistula* extract having lower FCR ratio (1.49±0.03). while in contrast the highest FCR values were recorded in 5th week in positive control group U (2.54±0.13) followed by Group E (2.37±0.45) treated with (10%) of *S. cumini* crude extract. (Table 4.5).

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Table 4.3: Effect of experimental plants crude extracts and their anticoccidial active fractions on mean weight gain of chicks in different experimental groups

Time	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K
3rd Week	232±2.6^{fg}	257.6±3.05^{e}	268.6±1.52^{de}	278.33±1.56^{cd}	224±1.56^{g}	229.6±2.45^{fg}	228.3±1.54^{fg}	226.45±3.24^{fg}	258.3±0.57^{e}	261.6±1.15^{e}	262.3±2.31^{e}
4th Week	304±6.2^{h}	317.3±2.5^{fg}	318.6±1.53^{ef}	327.3±1.56^{cdef}	318.3±2.57^{ef}	322.3±1.56^{def}	301.45±2.16^{h}	306.3±1.154^{fg}	318.3±1.15^{ef}	320.6±1.45^{ef}	321.3±1.15^{def}
5th Week	376.3±1.15^{g}	419.3±0.56^{cde}	421.5±2.34^{cd}	424.6±2.45^{c}	406.3±1.67^{f}	417.7±3.5^{cde}	385.3±2.15^{g}	385.2±1.73^{g}	415.2±2.13^{def}	421.3±0.54^{cd}	423.3±4.12^{cd}

Time	Group L	Group M	Group N	Group O	Group P	Group Q	Group R	Group S	Group T	Group U
3rd Week	269.6±4.0^{de}	287.3±1.15^{bc}	288.3±1.56^{bc}	230.33±2.56^{fg}	226.3±2.34^{fg}	227.6±4.5^{fg}	238.6±2.31	300.6±2.51^{ab}	311.6±3.05^{a}	148.2±1.56^{h}
4th Week	329.6±2.1^{cde}	337.3±1.56^{bc}	336.3±3.05^{bc}	323.7±2.61^{def}	326.3±2.34^{cdef}	328.6±2.6^{cdef}	332.6±1.15^{bcd}	343.6±2.08^{b}	392.3±2.08^{a}	229.3±4.04i
5th Week	426.3±1.5^{c}	444.3±2.03^{b}	447.3±1.5^{ab}	411.3±1.45^{ef}	411±1.76^{ef}	425.3±3.5^{c}	421.51±3.53^{cd}	443.1±3.21^{b}	456.3±2.51^{a}	277.6±3.78^{h}

Table 4.4: Effect of experimental plants crude extract and their anticoccidial active fractions on mean feed consumption of chicks in different experimental groups

Time	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K
1st week	402.6±0.5^{h}	409.3±0.5^{g}	417.6±1.15^{f}	422.6±2.1^{e}	396.3±1.15^{i}	402.3±1.52^{h}	411.3±2.51^{g}	416.3±1.32^{f}	411.3±1.5^{g}	417.6±2.08^{f}	427.3±0.57^{de}
2nd week	541.3±1.5^{ij}	548.6±0.6^{gh}	553.3±0.57^{ef}	555.3±1.5^{e}	538.1±2.31^{jj}	542.3±2.1^{ij}	547.6±1.15^{gh}	554±1.31^{ef}	547.3±1.52^{gh}	550.3±0.57^{fg}	564.3±0.56^{cd}
3rd week	690.3±0.5^{j}k	695.3±1.5^{hij}	704±1.71^{efg}	707±1.45^{def}	692.6±2.31^{j}	695.3±0.57^{hij}	693.6±0.57^{ij}	696.0±2.13^{hij}	697.6±1.34^{ghij}	702.6±0.57^{fgh}	713.3±2.51^{cd}

Time	Group L	Group M	Group N	Group O	Group P	Group Q	Group R	Group S	Group T	Group U
3rd week	430.6±0.57^{cd}	436.6±0.57^{b}	434.6±0.67^{bc}	365.6±1.15^{k}	378.6±2.51^{j}	413±2.51fg	416.6±2.08f	436.6±0.57^{b}	479.3±0.62^{a}	396±1.72^{i}
4th week	567.3±0.57^{c}	574.3±0.56^{b}	576.3±1.43^{b}	525.6±1.62^{k}	545.3±0.57^{hi}	548±1.72g^{h}	562.6±1.62^{d}	577.6±2.31^{b}	609.6±0.57^{a}	505.3±2.34^{l}
5th week	715.3±0.62^{c}	759.6±5.03^{a}	761±2.13^{a}	684.3±2.34^{kl}	696±3.21^{hij}	701.3±0.56fg^{hi}	710.6±1.32c^{de}	725.6±1.15^{b}	762.6±1.15^{a}	678±1.73^{l}

Table 4.5: Effect of experimental plants crude extract and their anticoccidial active fractions on mean feed conversion ratio of chicks in different experimental groups.

Time	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K
3rd week	1.6±0.01^{cde}	1.5±1.12^{cde}	1.5±1.41^{ef}	1.4±1.32^{ef}	1.9±1.15^{b}	1.81±0.01^{bcd}	1.6±1.45^{cde}	1.7±1.31^{cde}	1.5±1.14^{cde}	1.5±3.12^{ef}	1.6±1.31^{cde}
4th week	1.74±0.01^{efg}	1.69±2.13^{efg}	1.6±0.02^{fg}	1.58±1.41^{fg}	2.2±0.29^{ab}	1.83±1.31^{cde}	1.73±1.3^{efg}	1.6±2.21^{efg}	1.69±1.02^{efg}	1.6±1.41^{efg}	1.7±1.51^{efg}
5th week	1.8±1.23^{de}	1.7±2.13^{de}	1.64±1.31^{e}	1.6±0.05^{de}	2.37±0.45^{ab}	1.8±2.41^{cde}	1.8±1.31^{de}	1.7±3.1^{de}	1.77±2.1^{de}	1.7±2.1^{de}	1.69±2.12^{de}

Time	Group L	Group M	Group N	Group O	Group P	Group Q	Group R	Group S	Group T	Group U
1st week	1.5±3.1^{ef}	1.6±2.31^{cde}	1.2±2.13^{g}	2.2±1.34^{a}	1.9±0.01^{b}	1.8±1.12^{bc}	1.71±1.31^{cde}	1.2±0.20^{g}	1.3±0.12^{fg}	2.4±1.56^{a}
2nd week	1.7±0.07^{efg}	1.5±1.31^{fg}	1.56±1.54^{g}	2.03±1.7^{bc}	1.98±1.21^{cd}	1.79±0.10^{def}	1.7±1.31^{defg}	1.5±1.31^{fg}	1.5±0.002^{fg}	2.4±1.4^{a}
3rd week	1.6±1.56^{de}	1.5±2.41^{e}	1.5±1.2^{e}	2.1±1.17^{bc}	1.9±0.21^{cd}	1.8±1.41^{cde}	1.7±0.01^{de}	1.66±2.13^{de}	1.6±1.2^{de}	2.5±2.1^{a}

Effect of C. *fistula*, *F. racemosa* and *S. cumini* extracts and their anticoccidial active fractions on oocyst count per gram of faeces of chicks infected with mixed *Eimeria species*

The effect of selected plants crude extracts different concentrations, their anticoccidial active fractions, amprolium drug on oocysts counts of chicks infected with *Eimeria* species were investigated during first two weeks after infection. In all infected groups highest oocyst count were recorded at 7th and 8th day post infection. Gradual decrease in oocyst per gram of faeces was observed for all treatment groups except infected non-medicated positive control group U which showed significant increase. Maximum reduction was recorded in the group supplemented with amprolium having total oocyst count of (20.67) from 6th to 10th day post inoculation showing reduction from 7.33 to 4.13. Followed by group M (20.67) treated with 10% of *C. fistula* F-22, group N (21.00) treated with 20% of *C. fistula* F-22 and group D (21.33) treated 20% *C. fistula* crude extract. While the highest oocysts count was recorded in positive control group U *i.e.,* (38.33) followed by group R (31.00) treated with 20% of the *S. cumini* fraction 19 (Table 4.6).

Effect of *C. fistula*, *F. racemosa* and *S. cumini* extracts and their anticoccidial active fractions on bloody diarrhea, lesion score, mortality and survival rate of chicks infected with mixed *Eimeria species*

From 4th to 7th day after challenged infection with *Eimeria* species bloody diarrhea of all groups except negative control group was observed. The extent of bloody diarrhea was milder in group S supplemented with Amprolium 1.25*mg/ml*, followed by group M treated with CF Fraction 22 (10%), N treated with CF Fraction 22 (20%), J treated with 20% of *F. racemosa* fraction 19 and etc. Severe diarrhea was observed on 5th day of infection in positive control group U, group O treated with 10% of fraction 16 and P treated with 20% of fraction 16 of *S. cumini* leaves extract. On 7th day post infection bloody diarrhea disappears in majority of experimental groups while in few groups there is mild bloody diarrhea. No lesion score was observed in negative control group T, S medicated with amprolium 1.25*mg/ml* , C treated with *C. fistula* 10%, D

treated with *C. fistula* 20%, K treated with *C. fistula* Fraction 20 (20%), L treated with CF Fraction 20 (20%), M treated with CF Fraction 22 (10%), N treated with CF Fraction 22 (20%). Lesion score of +4 was recorded in positive control group U, O treated with *S.Cumini* Fraction 16 (10%), P treated with *S. Cumini* Fraction 16 (20%).

Table 4.6: Effect of experimental plants crude extract and their anticoccidial active fractions oocyst count per gram of faeces of chicks in different experimental groups

Days	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K	Group L	Group M	Group N	Group 0	Group P	Group Q	Group R	Group S	Group T	Group U
6th day	3.67	3.33	2.33	2.33	4.00	3.00	2.67	2.33	2.67	2.33	2.33	2.00	2.00	2.33	2.33	2.33	2.67	3.33	1.67	0.00	4.00
7th day	5.33	4.67	4.67	3.67	4.67	6.33	5.33	4.33	4.67	4.67	4.33	4.33	3.67	4.33	4.33	4.67	4.33	4.33	3.67	0.00	7.33
8th day	10.33	10.67	7.33	6.67	8.00	7.67	10.67	11.67	8.00	8.33	8.67	8.67	7.33	6.67	9.67	9.67	10.67	11.67	7.33	0.00	12.67
9th day	6.67	7.67	6.67	6.33	7.67	7.67	7.33	8.33	6.33	5.67	6.33	6.00	5.67	5.33	5.67	6.33	7.33	8.33	5.67	0.00	9.67
10th day	3.67	3.33	2.33	2.33	4.00	3.00	2.67	2.33	2.67	2.33	2.33	2.00	2.00	2.33	2.33	2.33	2.67	3.33	2.33	0.00	4.67
Total	29.67	29.67	23.33	21.33	28.33	27.67	28.67	29.00	24.33	23.33	24.00	23.00	20.67	21.00	24.33	25.33	27.67	31.00	20.67	0.00	38.33

The mortality was observed in majority of groups. Maximum mortality was seen in positive control group the infected non-medicated control group (50%), followed by (40%) in group O and P, G, H, A and B. No mortality was observed in groups C, D, K-N, S and T.

Throughout the experiment survival rate of chicks exposed to different concentrations of selected plants crude extracts and their anticoccidial active fractions as treatment of coccidiosis was observed. All treated groups had higher survival rate as compared to infected, non-medicated control group. 100% survival rate was shown by group C, D, K-N, S and T. Infected non-medicated group U showed 50% survival rate while all members of negative control group survived till the end of experiment (Table 4.7).

Effect of C. *fistula*, *F. racemosa* and *S. cumini* extracts and their anticoccidial active fractions on serum biochemistry of chicks infected with mixed *Eimeria species*

A significant increase in plasma ALT concentration was observed in chicks of infected non-medicated positive control group U (13.30±0.01) IU/L when compared with negative control group T (5.50±0.21) *IU/L.* The normal level of ALT is recorded in chicks of groups K(5.76±0.01), L(5.67±0.26), M(5.46±0.13), N(5.43±0.025) and in medicated group S(5.56±0.01) *IU/L*. Chicks infected with coccidian parasites revealed a highly significant increase in serum AST as compared with control group. The AST level increase significantly in infected un medicated positive control group U (313.9±5.03) U/l as compared to healthy negative control group T (283.49±2.71) U/l and medicated group S (275.9±3.43) U/l . The AST level was normal in groups C(297±2.56), D(292±3.16), K(288±1.56), L (285±2.13), M(278±1.56) and N(280±2.15).

The ALP level of chicks best treated groups C(300.3±0.87), D(304.29±3.36), K (309.27±1.53), L(309.34±2.69), M (307.58±3.5), N(318.54±1.13) U/l treated with *C. fistula* crude extract (10%), *C.fistula* crude extract (20%), fraction 20 of *C. fistula* (10%) and (20%), fraction 22 of *C. fistula* (10%) and (20%) respectively, were recorded normal and lies in the close range of negative control group T (325.33±2.47)U/l and

medicated group S (320.64±0.49) U/l . While the ALP level in remaining groups decreased from normal level.

The level of creatinine recorded normal in groups C (1.03±0.04), D(0.97±0.01), K (0.95±0.03), L(0.97±1.45), M (0.90±0.01), N(0.89±0.02) mg/dl treated with *C. fistula* crude extract (10%), *C.fistula* crude extract (20%), fraction 20 of *C. fistula* (10%) and (20%), fraction 22 of *C. fistula* (10%) and (20%) respectively, were recorded normal and lies in the close range of negative control group T (0.86±0.02) mg/dl and medicated group S (0.91±0.02) mg/dl. In contrast in remaining groups the creatinine level increased abnormally from normal range due to severe infection in these groups.

A significant increase in uric acid concentration was observed in infected non-medicated positive control group U (8.57±0.04) mg/dl when compared with uninfected negative control group T (6.24±0.52) mg/dl. The normal level of uric acid was recorded in groups C (6.36±0.01), D (6.32±0.01), K (6.36±0.02), L(6.32±0.01), M (6.26±0.01), N(6.26±0.02) and in medicated groupa S (6.27±0.02) mg/dl treated with *C. fistula* crude extract (10%), *C.fistula* crude extract (20%), fraction 20 of *C. fistula* (10%) and (20%), fraction 22 of *C. fistula* (10%) and (20%) respectively.

A significant decrease in albumin concentration was observed in positive control group U (0.72±0.03) as compared to negative control group T (1.29±0.08) gm/dl. The normal level of albumin was recorded in groups C (1.13±0.03), D(1.16±0.04), K (1.23±0.01), L(1.22±0.05), M (1.24±0.37), N(1.26±0.07) and in medicated group S (1.28±1.03) mg/dl treated with *C. fistula* crude extract (10%), *C.fistula* crude extract (20%), fraction 20 of *C. fistula* (10%) and (20%), fraction 22 of *C. fistula* (10%) and (20%) respectively (Table 4.8).

Table 4.7: Effect of experimental plants crude extract and their anticoccidial active fractions on bloody diarrhea, lesion score, mortality and survival rate of chicks in different experimental groups.

Groups	Bloody Diarrhea (Days)					Lesion Score	Mortality rate %	Survival rate %
	3rd	4th	5th	6th	7th			
A	-	+	++	+	+	3	40	60
B	-	+	++	+	-	3	40	60
C	-	+	++	+	-	0	0	100
D	-	+	++	-	-	0	0	100
E	-	+	++	+	-	2	20	80
F	-	+	+	+	-	2	20	80
G	-	+	++	+	+	3	40	60
H	-	+	++	+	+	3	40	60
I	-	+	+	-	-	2	20	80
J	-	+	+	-	-	2	20	80
K	-	+	++	+	-	0	0	100
L	-	+	+	-	-	0	0	100
M	-	+	+	-	-	0	0	100
N	-	+	+	-	-	0	0	100
O	-	+	+++	++	-	4	40	60
P	-	+	++	+	-	4	40	60
Q	-	+	+	+	-	3	20	80
R	-	+	+	+	-	3	20	80
S	-	+	+	-	-	0	0	100
T	-	-	-	-	-	0	0	100
U	-	++	+++	++	+	4	50	50

Table 4.8: Effect of experimental plants crude extract and their anticoccidial active fractions on biochemistry of blood of chicks in different

experimental groups

Parameters	Group A	Group B	Group C	Group D	Group E	Group F	Group G
ALT	6.79 ± 1.34^{f}	6.44 ± 2.56^{hi}	6.16 ± 2.31^{jk}	6.06 ± 2.41^{k}	11.7 ± 1.76^{b}	10.2 ± 3.12^{d}	6.55 ± 2.56^{gh}
AST	296.8 ± 1.19^{def}	289.8 ± 2.13^{ghi}	297.5 ± 2.34^{cde}	292.26 ± 1.56^{efgh}	307.51 ± 2.34^{ab}	303.5 ± 1.56^{bc}	290.86 ± 2.45^{fgh}
ALP	277.7 ± 2.34^{hij}	283.0 ± 2.5^{fgh}	300.2 ± 1.32^{d}	304.2 ± 2.31^{cd}	259.2 ± 1.45^{m}	269.4 ± 2.32^{kl}	280.6 ± 1.2^{ghi}
Creatinine	1.06 ± 1.03^{bc}	1.0 ± 2.13^{bfgh}	1.03 ± 1.45^{bcd}	0.97 ± 0.12^{bcd}	1.06 ± 1.71^{bc}	1.0 ± 2.31^{bc}	1.03 ± 1.23^{bcd}
Uric Acid	6.4 ± 0.01^{g}	6.4 ± 1.41^{gh}	6.3 ± 0.01^{hi}	6.3 ± 1.23^{ij}	7.1 ± 2.01^{b}	7.13 ± 1.42^{b}	6.7 ± 2.31^{e}
Albumin	1.09 ± 0.01^{ef}	1.08 ± 1.21^{ef}	1.13 ± 1.03^{cdef}	1.16 ± 4.12^{bcde}	1.04 ± 1.23^{f}	1.0 ± 1.15^{ef}	1.1 ± 1.02^{cdef}

Parameters	Group H	Group I	Group J	Group K	Group L	Group M	Group N
ALT	6.2 ± 1.6^{jk}	6.65 ± 2.45^{fg}	6.28 ± 1.62^{ij}	5.76 ± 2.45^{l}	5.6 ± 1.52^{lm}	5.46 ± 3.12^{n}	5.4 ± 3.12^{n}
AST	287.9 ± 1.67^{ghi}	292.21 ± 1.42^{efgh}	291.94 ± 1.56^{efgh}	288.5 ± 1.25^{ghi}	285.96 ± 1.45^{hij}	278.5 ± 1.23^{kl}	280.5 ± 0.92^{jkl}
ALP	284.4 ± 2.42^{efg}	287.8 ± 1.45^{ef}	289.3 ± 2.31^{e}	309.7 ± 1.5^{c}	309.3 ± 2.14^{c}	307.5 ± 2.13^{c}	318.5 ± 3.2^{b}
Creatinine	1.0 ± 3.42^{bc}	1.05 ± 3.14^{bc}	1.07 ± 1.56^{bc}	0.9 ± 2.31^{bcd}	0.97 ± 0.14^{bcd}	0.9 ± 1.56^{cd}	0.8 ± 2.14^{cd}
Uric Acid	6.7 ± 0.01^{ef}	6.71 ± 1.34^{ef}	6.64 ± 2.32^{f}	6.3 ± 3.12^{hi}	6.3 ± 1.34^{ij}	6.2 ± 1.32^{j}	6.2 ± 2.13^{j}
Albumin	1.12 ± 1.24^{def}	1.13 ± 0.03^{cdef}	1.12 ± 2.13^{def}	1.23 ± 1.46^{abcd}	1.2 ± 2.13^{abcd}	1.2 ± 1.32^{abc}	1.2 ± 1.26^{ab}

Parameters	Group O	Group P	Group Q	Group R	Group S	Group T	Group U
ALT	11.0 ± 1.24^{c}	10.96 ± 1.34^{c}	10.17 ± 3.12^{de}	10.0 ± 2.13^{e}	5.5 ± 1.71^{mn}	5.5 ± 2.12^{mn}	13.3 ± 2.61^{a}
AST	300.9 ± 1.56^{cd}	296.3 ± 2.13^{def}	296.88 ± 3.12^{def}	294.12 ± 132^{efg}	275.9 ± 2.13^{l}	283.4 ± 2.13^{ijk}	313.9 ± 1.52^{a}
ALP	265.6 ± 3.1^{l}	267.8 ± 1.24^{kl}	272.1 ± 2.31^{jk}	275.6 ± 3.24^{ij}	320.6 ± 2.13^{ab}	325.3 ± 2.56^{a}	257.6 ± 1.42^{m}
Creatinine	1.0 ± 2.14^{bc}	1.0 ± 2.13^{bcd}	1.03 ± 3.41^{bcd}	1.09 ± 2.14^{b}	0.9 ± 1.67^{bcd}	0.86 ± 1.62^{d}	1.5 ± 2.14^{a}
Uric acid	6.99 ± 1.54^{c}	6.97 ± 1.45^{cd}	6.9 ± 2.31^{cd}	6.8 ± 2.14^{d}	6.27 ± 1.02^{j}	6.2 ± 0.51^{j}	8.57 ± 1.45^{a}
Albumin	1.11 ± 1.21^{ef}	1.1 ± 1.42^{ef}	1.15 ± 2.14^{bcdef}	1.13 ± 1.04^{cdef}	1.2 ± 0.06^{a}	1.2 ± 0.18^{a}	0.72 ± 1.71^{fg}

Intestinal digesta viscosity

Among all groups intestinal digesta viscosity was higher in chicks of negative control group T (5.4±2.6) followed by group M (4.29±0.1) treated with 10% of fraction 22 of *C. fistula* extract and group N (4.17±2.1) treated with 20% of fraction 22 of *C. fistula* extract. The lowest intestinal digesta viscosity was observed in positive control group U (2.34±1.2) followed by group A (2.29±1.32) treated with 10% of *F. racemosa* crude extract followed by group B (2.88±1.67) treated with 20% of *F. racemosa* crude extract (Figure 4.1).

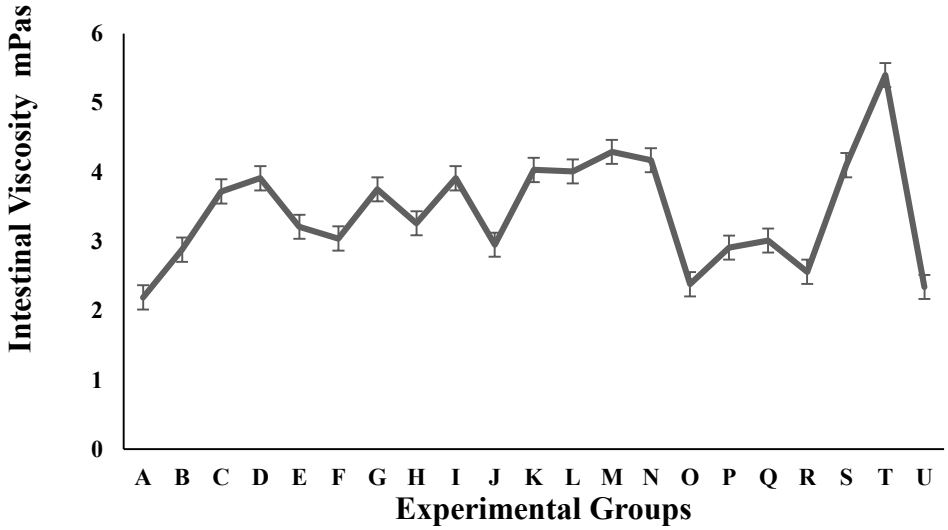


Figure 4.1: Effect of experimental plants crude extract and their anticoccidial active fractions on intestinal digesta viscosity of chicks in different experimental groups.

Complete blood count (CBC) (Hematology)

Haemoglobin (g/dL)

The best experimental groups showed similar haemoglobin level to the negative control group T (11.34±2.37) normal range of haemoglobin level *i.e.*, C(10.76) treated with 10% of *C. fistula*, D(10.98) treated with 20% of *C. fistula*, K(11.0) treated with 10% of *C. fistula* F-20, L(11.2) treated with 20% of *C. fistula* F-20, M(12.0)treated with 10% of *C. fistula* F-22, N(11.6) treated with 20% of *C. fistula* F -22. While a significant decrease in haemoglobin level occur in positive control group U (6.56±2.511), group

E(7.9±1.45) treated with 10% *S. cumini* and F (7.5±2.34) treated with 20% *S. cumini* crude extracts. A highly statistically significant ($P < 0.01$) decrease in packed cell volume level was evident in group Q (21.5±2.15) treated with 10% of *S.cumini* F-19 and group E (22.6±1.45) treated 10% of *S.cumini* crude extract similar to the positive control group U (18.5±1.65). while the normal range of PCV was observed in negative control group T (49.5±3.15), group L (48.9±2.45) treated with 20% of *C. fistula* F-20 followed by chicks of group N having normal PCV (43.5±1.56) treated with 20% of *C. fistula* F-22. Normal range of RBCs and WBCs observed in groups C, D, K, L, M, N, medicated group S and in negative control group T. Decrease in total RBCs count and increase in WBCs was observed in positive control group U and in some other crude extracts and fractions treated groups. A total significant decrease in lymphocytes concentration was observed in positive control group U, E, Q treated with 10% *S.cumini* crude extract and 10% of *S.cumini* F-19 respectively. While their normal no was recorded in remaining groups just like negative control group T. Further hematological analysis revealed significantly lower ($P < 0.05$) levels of MCV (fL), MCH (pg) and MCHC (g/L) for chickens in positive control group U and in some other infected groups chicks compared with those levels in non-infected control chickens. The levels of these hematological parameters in chickens infected with *Eimeria* species were also below the normal ranges, whereas the levels in non-infected control chickens and in best treated groups were within the normal ranges (Table 4.9).

Table 4.9: Effect of experimental plants crude extract and their anticoccidial active fractions on hematology of chicks in different experimental groups.

Groups	WBC×(10^3/ul)	RBC×(10^6/ul)	HB (g/dL)	HCT (%)/(PCV)	MCV (fl)	MCH (pg)	MCHC (g/dL)	PLT(10^6/ul)	LYM (%)	RDW (fL)
A	16.2	2.3±1.59	8.6±2.13	36.5	131.5	35	29.7	3.9	14	10.7
B	16.9	2.5±2.31	8.8±0.11	29.8	136.7	37	31.2	2.5	12	10.3
C	11.5	2.7±1.05	10.76±1.53	37.9	137.5	29	30.3	2.9	31	11.6
D	11.8	2.7±1.56	10.98±2.13	36.1	139.4	30	30.7	2.22	35	10.3
E	19.2	2.1±2.13	7.9±1.60	22.6	134.1	44	28.5	3.1	10	9.4
F	19.6	2.1±1.03	7.5±2.31	27.4	121.7	41	29.6	3	15	11.9
G	13.03	2.5±1.55	9.4±1.56	33.5	135.4	44	32.8	1.7	13	13
H	13.5	2.6±2.41	9.7±2.31	35.2	137.9	43	30.6	2.6	18	10.3
I	15.2	2.5±1.33	9.5±1.24	30.4	131.1	40	28.7	3.7	14	12.5
J	17.4	2.7±0.00	9.7±2.31	33.3	109.2	31	30.7	2.1	12	11.1
K	13.9	2.8±1.05	11.01±1.51	39.7	122.3	33	32.6	3.1	25	10.3
L	13.4	2.8±2.13	11.23±2.13	48.9	139.5	31	31.5	3.3	24	10.1
M	10.7	3.1±1.02	12.03±2.41	40.7	138.6	30	30.4	2.6	29	11.1
N	10.9	2.3±1.31	11.57±0.02	43.5	138.9	32	29.3	2.8	33	11.16
O	18.5	2.2±2.13	8.9±1.01	32.7	130.7	44.1	31.2	2.3	27	11.7

P	18.2	2.4±1.03	8.9±0.31	33.8	117.5	36.9	30.6	1.6	18	9.6
Q	14.3	2.5±2.01	9.3±1.23	21.5	96.11	37	29.5	2.9	10	8.7
R	14.9	2.7±1.22	9.6±0.21	30.4	108.5	37	31.1	4.1	12	10.9
S	11.23	2.9±1.02	12.58±4.92	46.2	136.3	31	30.4	2.9	30	11.1
T	10.6	3.3±2.32	11.34±2.37	49.5	138.2	31.4	33.6	2.6	25	11.2
U	21.3	1.8±1.11	6.56±1.23	18.5	84.2	44.2	29.7	4.6	8	16.7

Evaluations of drug resistance

Anticoccidial index (ACI), anticoccidial sensitivity test (AST) and percent optimum anticoccidial activity (POAA).

ACI, AST and POAA are used for the evaluation of drug resistance. Mortality (40%) was recorded in some groups like A, B, G, H and in Q, P and S. maximum of (50%) mortality was recorded in infected untreated positive control group. The ACI values of all groups were (>160) except groups A (124.44), B(142.78),G(141.05), H(137.85), S(147.92) and U (74.67) indicated that the used extracts and their fractions were able to treat the chickens infected with different eimeria species. Resistance to amprolium treated group S was expressed as the value of S(147.92) (Table 4.10).

AST values (> 50%) for all groups except groups A (37.5) , O(12.5), P(20.6) and U(0.00) showed that *Eimeria* species are sensitive to the different extracts and their fractions tested, however, sensitivity to amprolium was much less as their AST value was (40.00) (Table 4.11). POAA values of all groups are (> 50%) except groups A, B, E, F, I, J, O and U. while amprolium treated group showed less sensitivity as POAA value (36.98) less than 50. In the present study, an overall drug sensitivity assessment based on a combined analysis of the three indexes revealed that group A and S showed strong resistance, B and Q showed moderate resistance, groups E-J, P showed slight resistance, and rest of the groups showed sensitivity to their respective treatments (Table 4.12).

Table 4.10: Results of Anti coccidial sensitivity test and anticoccidial index

Groups	Relative rate of weight gain	Survival rate	Lesion value	Oocysts value	ACI	AST
A	144.33	60	2.5	77.39	124.44	37.5
B	161.67	60	1.5	77.39	142.78	62.5
C	154.00	100	0.5	60.87	192.63	87.5
D	146.33	100	0.5	55.65	190.18	87.5
E	182.33	80	1	73.91	187.42	75
F	188.00	80	1	72.17	194.83	75
G	157.33	60	1.5	74.78	141.05	62.5
H	155.00	60	1.5	75.65	137.85	62.50
I	156.67	80	1	63.48	172.19	75
J	159.67	80	2	60.87	176.80	50
K	161.00	100	0	62.61	198.39	100
L	156.67	100	0	60.00	196.67	100.00
M	144.33	100	0	53.91	190.42	100.00
N	146.00	100	0	54.78	191.22	100.00
O	186.67	60	3.5	63.48	179.69	12.5
P	184.67	60	3.2	66.09	175.38	20
Q	197.67	80	1.5	72.17	203.99	62.5
R	187.67	80	1.5	80.87	185.30	62.50
S	142.33	60	2.4	53.91	146.02	40.00
T	144.67	100	0	0.00	244.67	100.00
U	128.67	50	4	100.00	74.67	0.00

Table 4.11: Results for percentage optimum anticoccidial (POAA)

Groups	Initial body weight	Final body weight	GSR	POAA %
A	1658.25	4579.23	2.76	27.10
B	1629.27	4321.2	2.65	-24.50
C	1798.36	5499.08	3.06	167.06
D	1797.23	5584.15	3.11	190.33
E	1685.24	4629.15	2.75	20.20
F	1676.23	4674.31	2.79	39.90
G	1693.71	4785.28	2.83	57.25
H	1729.55	4945.37	2.86	73.32
I	1745.24	4529.19	2.60	-51.45
J	1791.91	4934.46	2.75	23.44
K	1903.82	5711.62	3.00	139.79
L	1945.57	5917.41	3.04	159.34
M	1987.41	5981.35	3.01	144.30
N	1826.38	5812.77	3.18	226.03
O	1623.41	4534.33	2.79	42.03
P	1745.93	4967.25	2.85	66.57
Q	1724.28	4912.71	2.85	68.50
R	1641.56	4823.27	2.94	110.57
S	2011.73	5597.46	2.78	36.98
T	2067.34	6028.02	2.92	100
U	1525.16	4124.19	2.70	0

Table 4.12: Overall assessment of the sensitivity of *Eimeria* species

Groups	ACI	AST	POAA%	Resistance
A	+	+	+	strong
B	+	-	+	Moderate
C	-	-	-	None
D	-	-	-	None
E	-	-	+	Slight
F	-	-	+	Slight
G	+	-	-	Slight
H	+	-	-	Slight
I	-	-	+	Slight
J	-	-	+	Slight
K	-	-	-	None
L	-	-	-	None
M	-	-	-	None
N	-	-	-	None
O	-	+	+	Moderate
P	-	+	-	Slight
Q	-	-	-	None
R	-	-	-	None
S	+	+	+	strong
T	-	-	-	None
U	+	+	+	Strong

Organ weight

The higher mean organ weight values were observed in all treated groups (P<0.05) than infected non-medicated control group. Among all groups on 36th day of age the mean internal organ weight relative to the body weight was remain normal in groups i.e., C, D, K, L, M, N and in healthy negative control group T. In some groups i.e., group U positive control group, E-G and group Q the increased in weight of liver recorded and this is due to hypertrophy. Similarly, in case of intestine, in some infected groups a slight increase in normal weight was observed due to hypertrophy in intestine also (Table 4.13).

Table 4.13: Effect of experimental plants crude extract and their anticoccidial active fractions on percent organ weights ratio relative to live body weight of experimental groups.

	Organs	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**
Percent organ relative weight	**Liver**	4.27	4.22	3.11	3.09	5.61	5.12	5.34	4.76	4.31	3.71	3.9	3.06	3.02	3.02	3.19	3.25	5.14	4.79	2.98	3.56	5.61
	Spleen	0.17	1.9	0.15	0.15	0.17	1.5	0.17	0.17	0.19	0.16	0.16	0.14	0.14	0.15	0.16	0.18	0.17	0.17	0.14	0.15	0.2
	Intestine	14.78	15.29	14.91	13.27	15.62	16.01	14.27	14.36	11.25	14.9	12.57	12.32	13.28	11.21	13.26	12.34	11.25	12.71	14.06	13.22	15.02
	Bursa	0.33	0.35	0.25	0.23	0.32	0.29	0.25	0.19	0.21	0.17	0.34	0.29	0.36	0.25	0.17	0.26	0.23	0.27	0.25	0.26	0.17
	Kidney	1.18	1.21	0.15	0.19	0.25	0.91	0.54	0.71	0.63	0.76	0.17	0.13	0.15	0.17	0.25	0.37	0.49	0.47	0.11	0.7	1.24
	Heart	0.41	0.32	0.37	0.31	0.27	0.38	0.37	0.29	0.45	0.31	0.42	0.43	0.47	0.45	0.42	0.41	0.39	0.41	0.44	0.473	0.21

Pathomorphological alterations

Post mortem Observations of Organs

The internal organs of best treated groups *i.e.,* J, L, M, N,P, R , S and negative control group T are normal while the internal organs of remaining groups i.e., group A, B, E-J , positive control group U and C, D, K N are not normal. The intestine of chicks in these diseased groups llooking grossly, extremely ballooned and hemorrhages in intestinal mucosa were seen. Watery ingesta mixed with mucus and blood was observed. Hemorrhagic enteritis was seen in whole portion of intestine(Figure 4.2). The caecum was enlarged with clotted blood, hemorrhagic spots on caecal wall, inflammation, necrotic patches and dilation of caecum with consolidation of caecal contents were observed in almost all the groups except control. On opening the caeca, the bloody mass, a characteristic of caecal coccidiosis, was found in some cases. The change in color from red to mottled reddish or milky white due to formation of oocysts (Figure 4.3). The postmortem examination of liver revealed the darkening, increasing in size, swelling. The liver was found to be pale and enlarged (Figure 4.4).

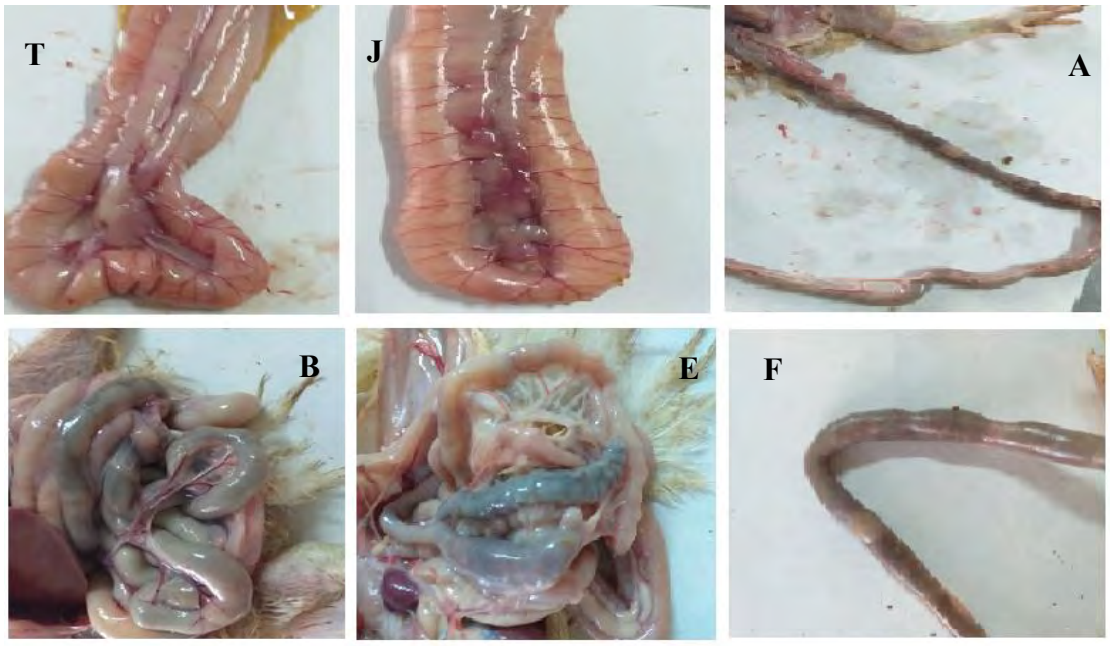


Figure 4.2: Postmortem observation of intestine of healthy, infected and recovered chicks.

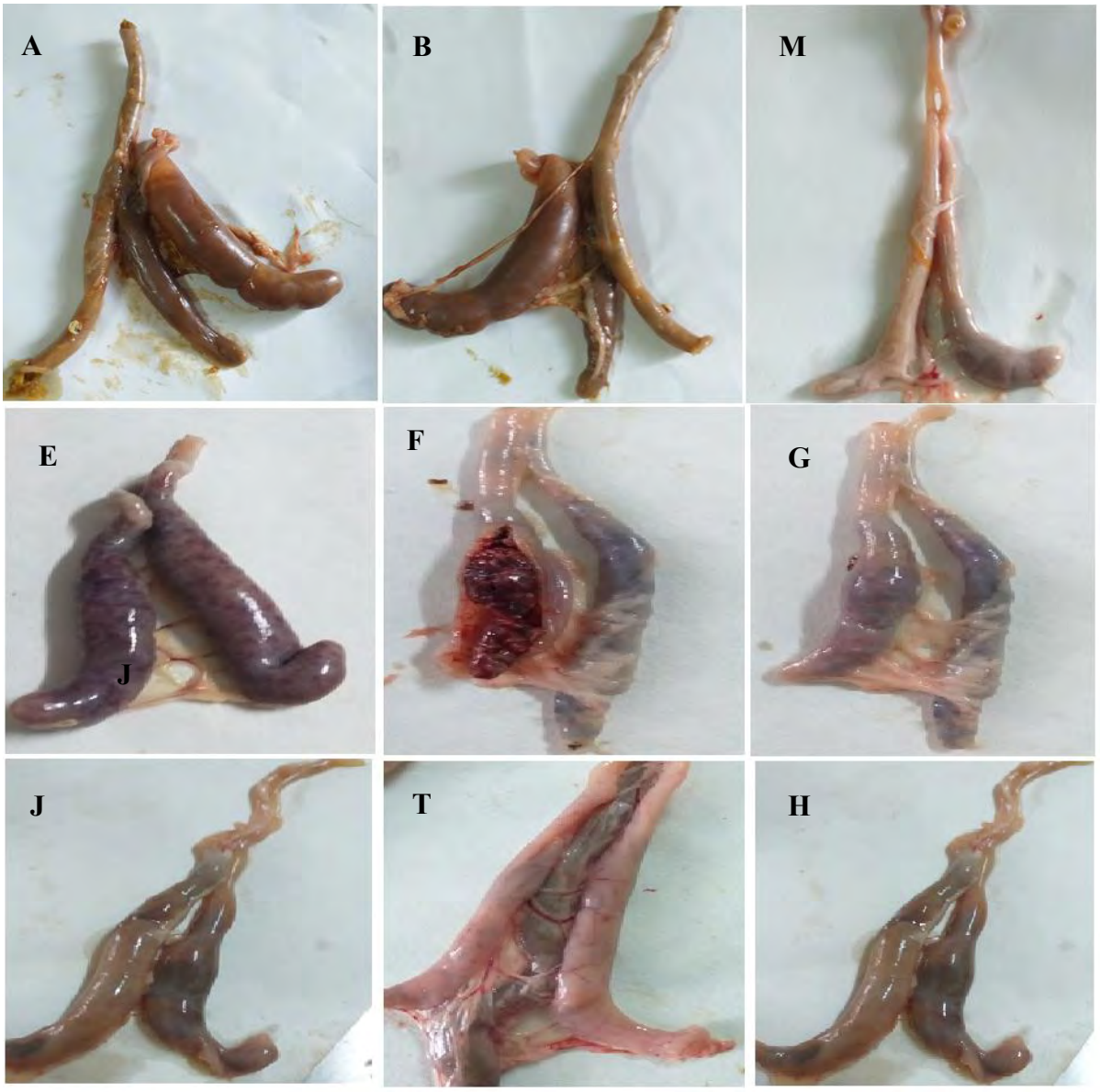


Figure 4.3: Postmortem observation of caecum of healthy, infected and recovered chicks.

Histopathological changes

Healthy groups *i.e.,* J, L, M, N,P, R , S and negative control group T showed normal histological structure of caecum mucosa, sub mucosa and lamina propria as compared to positive control group U and other experimental groups. While in caecum of remaining herbal treated and positive control U epithelial necrosis, merozoites in schizonts, cysts in sub mucosal glands was observed (Figure 4.5).

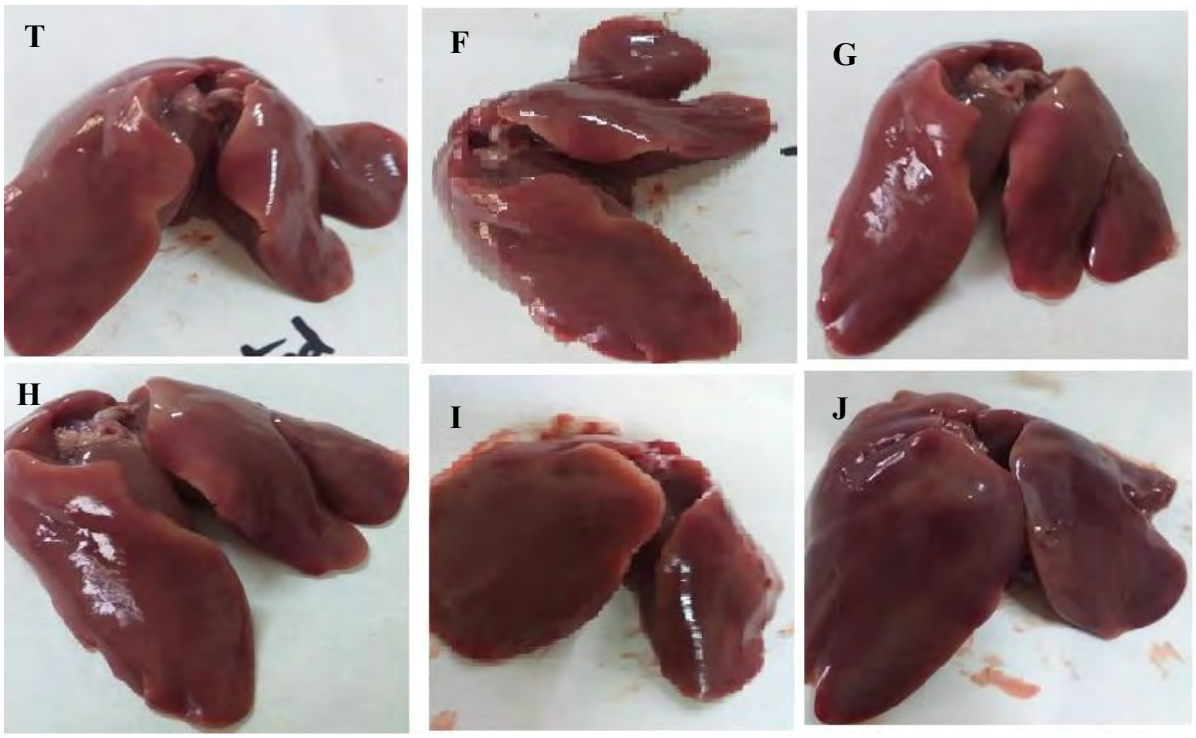


Figure 4.4: Postmortem observation of liver of healthy and diseased chicks

Normal histology of liver was observed in best treated groups *i.e*., J, L, M, N,P, R , S and negative control group T. While in remaining groups hepatic congestion, bi nucleated hhepatocytes and minimal necrosis of hepatocytes with infiltration with high congestion in central vein was observed. Substantial increase in normal hepatocytes size was observed in positive control group U, A, B, E-J. while the normal size of hepatocytes was observed in group C, D,K-N and in group S and T (Figure 4.6).

Duodenal glands in intestine of group A, B, C, D, E, and positive control group U having different parasitic stages, desquamated intestinal epithelium, hyperplasia of goblet cells, oocysts in epithelium, presence of intracellular developmental stages of *Eimeria* species are observed. While the intestinal histology was normal in best treated groups *i.e*., M,N, J, P, L, R and in negative control group T (Figure 4.7).

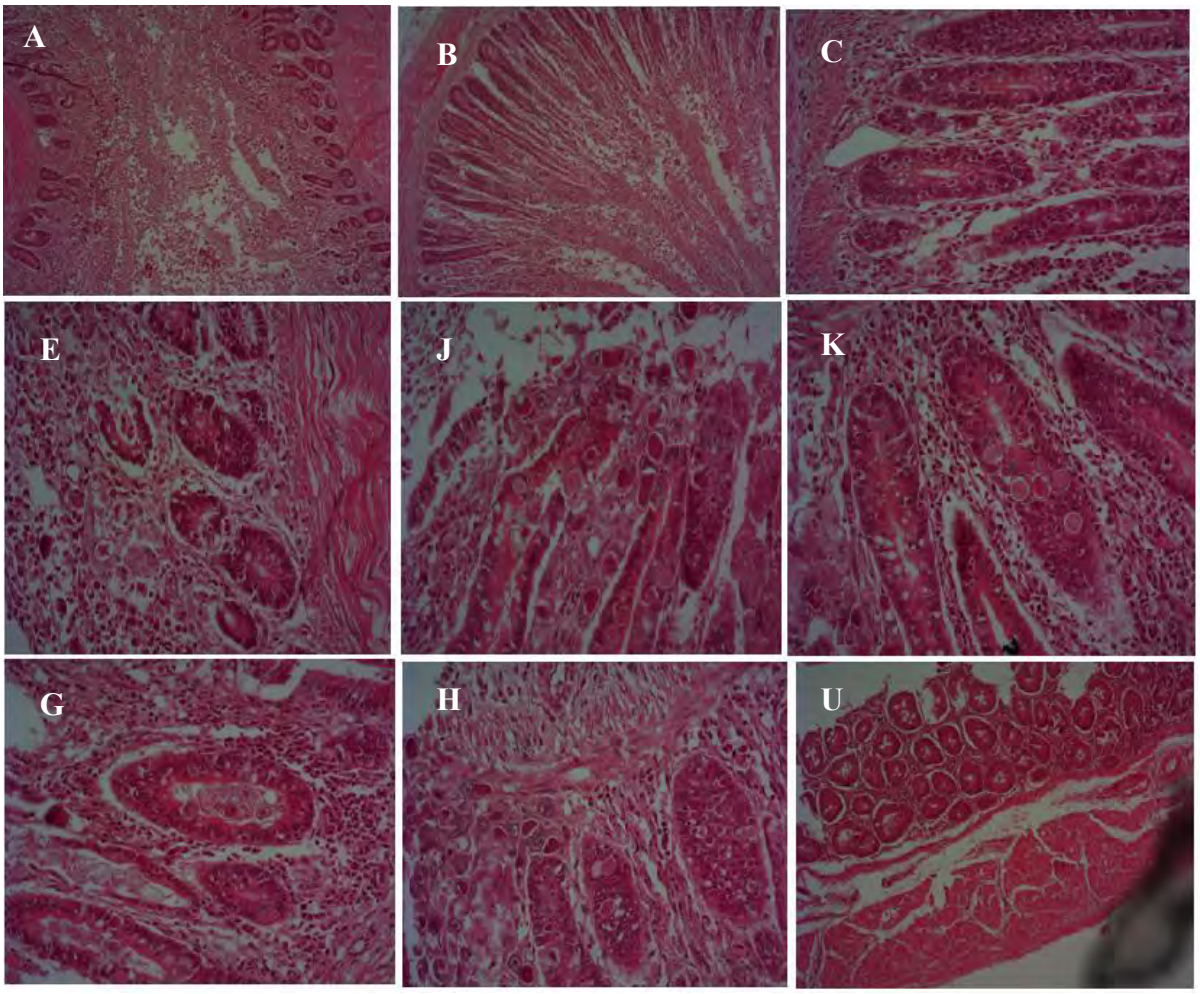


Figure 4.5: The histological studies of caeca of experimental treated animals VS control.

The histological study of kidneys of best treated groups i.e., M, N, J, P, L, R and negative control group T showed normal glomerular basement membrane, central vein, cortex and medulla. While the histology of the kidneys of other experimental groups showed tthickening of glomerular basement membrane, renal congestion and etc. Increased in Bowman s' Space occure in group U, A, B, E-J, O-R. while normal bowman s'space is present in group C, D, K-N, S and T. Renal corpuscular area reduce in size in group A, B, E,F, Q-R, and in group U.while remain normal in group C,D,K-N, and in S,T groups. Glomerular area reduce in size from normal in groups A, B, E, F, Q-R and in U. while the size of glomerular are remain normal in group C,D, K-N, S and T groups (Figure 4.8).

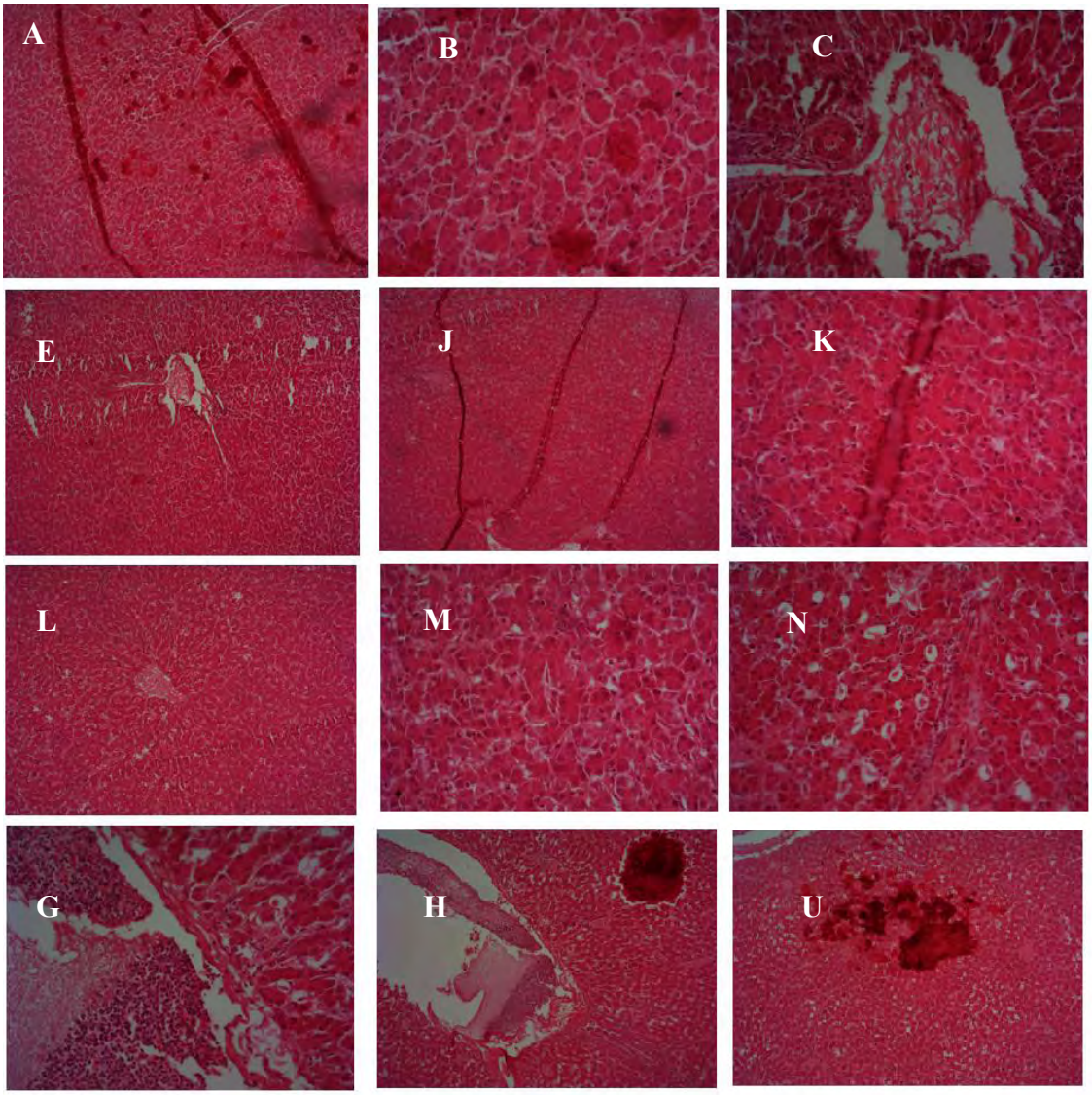


Figure 4.6: The histological studies of liver of experimental treated animals VS control.

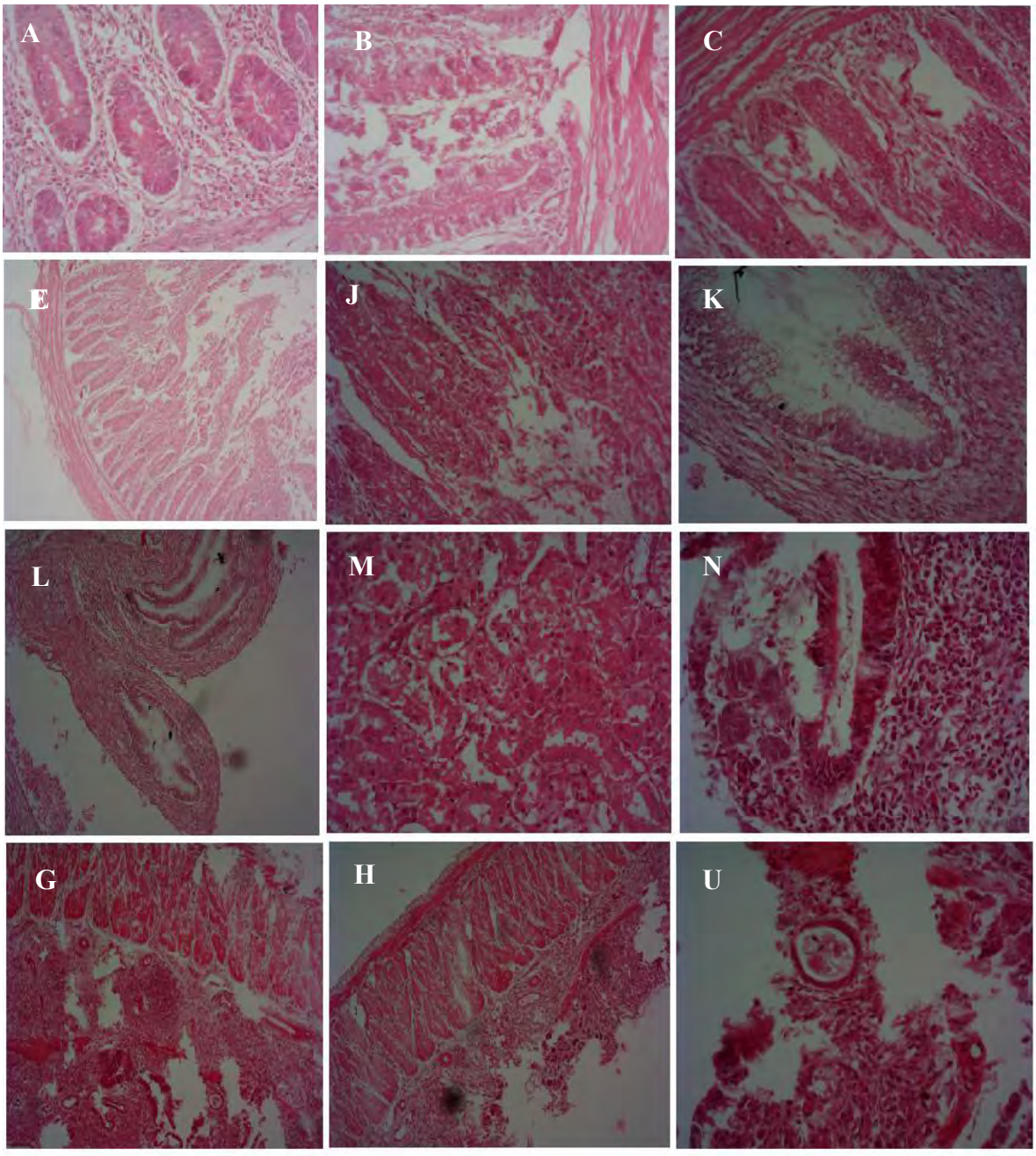


Figure 4.7: The histological studies of Intestine of experimental treated animals VS control.

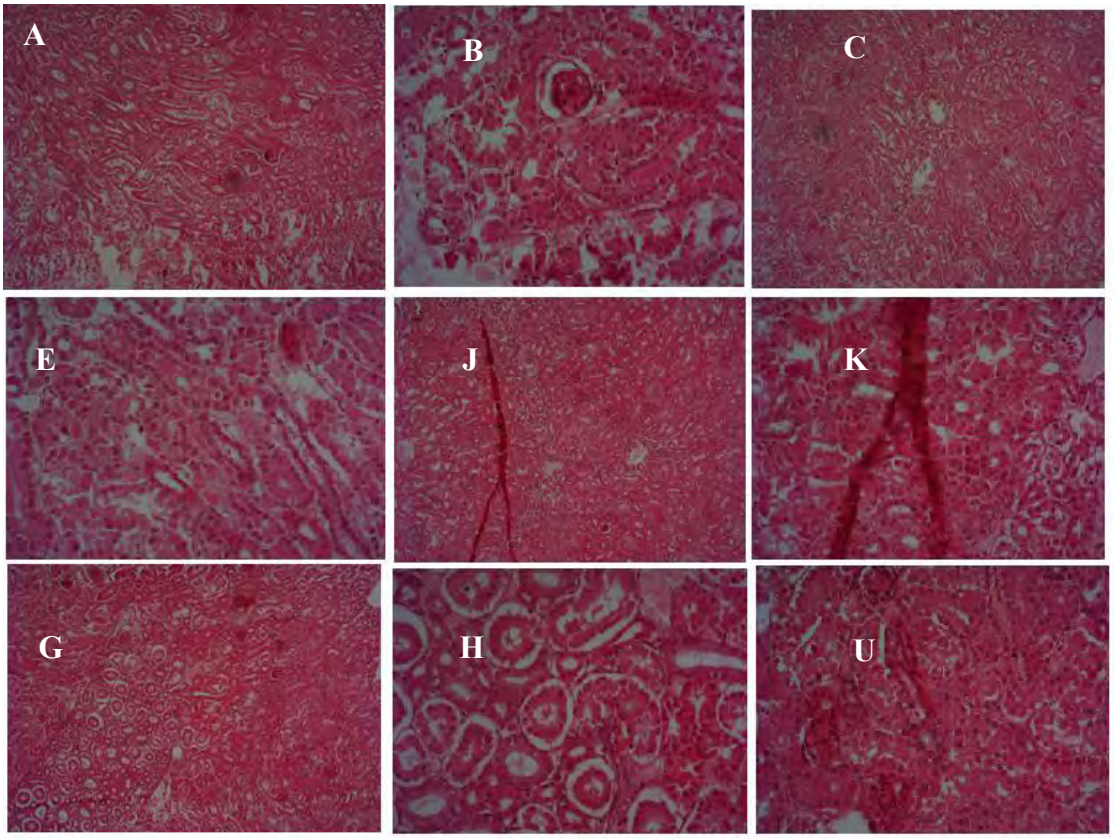


Figure 4.8: The histological studies of kidneys of experimental treated animals VS control.

DISCUSSION

It is essential to look for the treatment of *Eimeria* parasite because of its mortality and significant economic damage. Due to the excessive use of anticoccidial medications, the parasite has developed resistance to the available medications (Blake and Tomley., 2014). A cheap natural remedy for emerioisis that has fewer side effects is being investigated by experts. Natural anti-oxidants have recently drawn a lot of interest since they are safe, nourishing, and medicinal when taken in conjunction with herbs and other biological components (Goodla *et al*., 2019). According to reports, plants are a crucial source since they have always been used to cure a variety of disorders. Basir *et al.,* (2012) highlighted that *in vitro* research help to understand disease pathogenesis and therapy, but *in vivo* models are necessary for accurate physiological processes and a complete understanding of the body's chemistry. The present study was based upon the *in vivo* use of medicinal plants extracts and their fractions to cure coccidiosis. Broiler chicks were used as model animals.

The poultry industry has relied heavily on anticoccidial medications to prevent and treat avian coccidiosis, which has led to the emergence of *Eimeria* strains that are resistant to all anticoccidial medications that have been introduced (Abbas *et al*., 2011). Because of this, current research has concentrated on alternate methods of preventing avian coccidiosis, by using plants as anticoccidial agents (Abbas *et al*. 2010, Awais *et al*. 2011).

To assure the safety of usage, the toxicological evaluation of plant extract aims to identify any potential side effects. The toxicity of herbal extracts can be affected by several phytochemicals. This toxicity may come from the herbal plant components themselves or from the extraction method itself (Barros *et al*., 2005). No significant toxicity has been reported in acute toxicity experiment of selected experimental plants and their fractions. These findings are consistent with the study of Kumar and Sachin., (2013), on mice by checking the toxicity of 2000 *mg/kg* dose of *F.racemosa* methanolic extract and reported that all the animals were found healthy and the extracts were found to be safe up to a dose of 2000 *mg/kg*.

Similarly acute oral toxicity study of *C.fistula* methanolic extract with dose of 5000*mg/kg* on mice was conducted by Subramanion *et al*., (2011) resulted in no mortalities and behavioral changes in experimental animals. Siliva *et al*., (2012) determine LD_{50} of *Syzigium cumini* leaves extracts and proposed that there is no change in behavior and morphology of experimental animals.

By determining the effect of selected plants methanolic crude extracts and their fractions on mean body weight gain of chicks, maximum weight gain was recorded on 5th week in group N (447±4.04) and M (444±3.05) gm treated with (20%) and (10%) of F-22 of *C. fistula* respectively which was in higher than the weight gain of amprolium treated group. Similarly, a study conducted by Ibrahim *et al*., (2016) on anticoccidial effect of different fractions of *solanum dasyphyllum schumach* and *thonn* Crude methanolic extract observed that Fractions 6 and 9 of the six fractions of *S. dasyphyllum* were more efficient against sporulated oocysts of coccidia than amprolium.

Maximum mean feed consumption was recorded at 5th week of age in groups N (761±0.57) gm and M (759±0.57) gm treated with (20%) and (10%) of F22 of *C. fistula* respectively which was higher than the mean feed consumption of group treated with amprolium (725±1.52)gm and in the range of uninfected non-medicated control group (762±1.15) gm. While the feed consumption is much more less in on 3rd week in group P treated with (20%) of F16 of *S.cumini* similar to the positive control group U mean feed consumption i.e., (396±1.73)gm. Similarly, a study conducted by Christaki *et al*., (2004) proposed that Infection with *E. tenella* significantly reduced body weight gain and feed intake, and increased feed conversion ratio values highlighting the detrimental effect of the infection with this parasite on broiler performance. However, there were no significant differences between the Apacox levels for body weight gain, feed intake.

Among all experimental groups best FCR was shown by group N (1.25±0.01) treated with (20%) of fraction 22 of *C. fistula.* Followed by group D treated with (20%) of crude *C. fistula* extract (1.49±0.03). these values are in the range of amprolium medicated group S and negative control group T. From the time of infection to recovery due to lower feed consumption, feed conversion ratio of all infected groups were higher than uninfected non-medicated control group, as result all chicks lost weight. The

findings of the present study are similar to the work of Habibi *et al*., (2014) that the best treated groups showed lower FCR than other experimental groups.

Similarly Chandrakesan *et al*., (2009) observed that the herbal complex can cause better body mass gain and best FCR after infection with *E. tenella*. Similarly, according to Naidoo *et al*., (2008) the herbal extracts produced an improved FCR over the control in a similar manner to that for toltrazuril. The improved performance or FCR obtained by infected chicks fed with aqueous methanolic extracts, in the present study, might be a result of enhanced absorption of nutrients in intestinal mucosal surface by activity of different antioxidant compounds. Due to high surfactant activity of antioxidants, better absorption of nutrients by the intestinal epithelium is observed and thus improving FCR (McAllister *et al*., 1998). The other possibility of high absorption of nutrients may be due to natural ability of saponins in stabilizing the cell membranes by forming pores (Plock *et al*., 2001) and the beneficial effect on intestinal turnover as reported by Alfaro *et al*., (2007). Similar results on better FCR are also observed in previous studies (Chang *et al*., 2016).

Highest oocysts shedding was recorded on 7th and 8th day of infection in all infected groups. Gradual; decrease in oocysts shedding were observed in all groups except in positive control group U. among experimental groups maximum oocysts shedding was recorded by group R treated with 20% of the *S. cumini* fraction 19. Maximum reduction was recorded in the group supplemented with amprolium from 6th to 10th day post inoculation. Followed by group M treated with 10% of *C. fistula* F22, group N treated with 20% of *C. fistula* F-22 and group D treated 20% *C. fistula* crude extract. Abbas *et al.,* (2006) reported reduction in oocysts per gram using ethanolic and aqueous extracts of *Azadirachta indica* fruits and their powdered form. Also decreased oocyst excretion in *Schinopsis lorentzii* extract group in broiler chicks as compared to that of were challenged with mixed suspension of sporulated oocysts of *E. tenella*, *Eimeria maxima* and *Eimeria acervulina* were observed due to proanthocyanidins in the *S. lorentzii* extract that have potential effect against a mixed *Eimeria* challenge (Cejas *et al*., 2011).

After challenge infection from 4th to 7th day of infection bloody diarrhea in all groups except negative control group was observed. The milder bloody diarrhea was

observed in medicated group S followed by group M, N, J, etc. Severe diarrhea was observed on 5th day of infection in positive control group U, group O and P. On 7th day post infection bloody diarrhea disappears in majority of experimental groups while in few groups there is mild bloody diarrhea.

Similarly, a study conducted by Qaid *et al*., (2020) proposed that bloody diarrhea was observed in all infected, herbal treated groups. The bloody diarrhea of the infected birds was not affected in this trial, although reduced bleeding can protect infected birds against secondary infections, inflammatory response, and toxic substances absorption (Jamila *et al*., 2017). Similar results of mild bloody diarrhea was shown by study of Youn *et al.,* (2001) and Kostadinovic *et al.,* (2012).

No lesion score was observed in negative control group T, medicated group S , and in herbal extract treated group C, D, K , L, M ,N groups. Lesion score of +4 was recorded in positive control group U, O and in P. Coccidiosis is a deadly disease caused mortality in chicks (Fatoba and Adeleke., 2018) like approximately 50% mortality was recorded in positive control group followed by 40% mortality in group O and P, G, H, A and B. No mortality was observed in some best treated groups like C, D, K-N, S and T.

The decrease in lesion score severity might be due to its ability to depress oocyst multiplication as earlier observed in this study; it may also be associated with the amelioration of the deleterious effects of the parasitism probably through its antioxidant as well as free radical scavenging properties as observed by earlier workers (Samir *et al*., 2010). The observation of attenuation of the caeca lesion in this study is similar to the finding of Lee *et al*., (2013) which revealed that *P. guajava* leave effectively reduced lesions by another coccidian.

Results of current study show that all plant extracts reduced lesion scores, oocyst, fecal scores and oocysts per gram of feces levels in infected chicks. The positive effect on these parameters may be due to the antioxidant compounds as they by ameliorate the degree of intestinal lipid peroxidation and thus reduce the severity of *Eimeria* infections (Allen *et al*., 1997). Moreover, it can be emphasized that plant extract suppressed development of *Eimeria* life cycle in the host cell before oocysts are released in feces of chicken thus ultimately decreased *Eimeria* oocyst excretion and

severity of infection (Alzahrani *et al*., 2016). Treatment of infected chickens with herbal complex also showed positive effects on these parameters (Zaman *et al*., 2012). Similar findings on these parameters are observed in previous studies (Lan *et al*., 2016).

Nidaullah *et al.,* (2010) and Ahmed *et al*., (2018) reported low mortality rate in chickens treated with different herbs than positive control group. In contrast, study of Naidoo *et al.,* (2008) and Zaman *et al.,* (2012) reported highest rate of mortality compared with control. In present study, all plant extracts reduced mortality rate in infected chicks. The decline in mortality rate of plant treated groups could probably be attributed due to disease prevention by these extracts. This might be due to the fact that antioxidant compounds act on the cholesterol present on the parasite/protozoal cell membrane and inhibit of protozoal development due to which death of parasite occurs (Lala *et al*., 2015). In a recent study, methanolic extract of *Bidens pilosa* reduced mortality rate in chickens infected with *Eimeria tenella* (Yang *et al*.,2015). Same results on mortality rate are observed in previous studies (Hema *et al*., 2015: Lala *et al*., 2015).

All treated groups had higher survival rate as compared to infected, non-medicated control group. 100% survival rate was shown by group C, D, K-N, S and T. Infected non-medicated group U showed 50% survival rate while all members of negative control group survived till the end of experiment. Similarly a study conducted by Song *et al*., (2020) proposed that the survival rate of chicks increase with the administration of herbal extracts of Shi Ying Zi in Chicks infected with *Eimeria tenella.*

Due to coccidia infection in chicks serum enzymes activity (AST and ALT) revealed significant increase ($P<0.05$) in some experimental groups than control group and some best treated groups like K, L, M, N treated with different concentrations of fractions of C. fistula. These results are in agreement with *Mondal et al.,* (2011) and *Hirani et al*., (2007) in coccidia infected broiler chicken. Increase in the levels of serum enzymes might be due to cellular damage particularly of hepatocytes. In contrast *Adamu et al.,* (2013) reported decrease in ALT and AST activities in broilers infected with *Eimeria Spp.*

The normal level of albumin was recorded in groups C, D, K , L, M ,N and in medicated group S. A significant decrease in albumin concentration was observed in positive control group U as compared to negative control group T and in some other

infected groups like A, B, E, F, G, H, I, J, O, P, Q, R. These results are in accordance with Mondal *et al*., (2011), Hirani *et al*., (2007), Patra *et al*., (2010). Marked reduction in values of serum albumin might be due to nutrient malabsorption, hepatocellular damage, marked haemorrhagic enteritis, kidney dysfunction and inappetence. While normal range of serum albumin level in groups M, N treated with *C. fistula* fraction 22 different concentrations, group C and D treated with *C. fistula* crud extract different concentrations, might be due to its strong anticoccidial, anti-inflammatory, immunomodulatory and hepato-renal protective action leading to repair of coccidia induced injury of visceral organs as was observed during gross and microscopic pathology in present study (Dar *et al*., 2014).

The level of ALP decreases in coccidia infected groups except the groups which were treated with *C. fistula* crude extracts and their fractions different concentrations i.e., groups C, D, K, L, M, N were recorded normal level of ALP and lies in the close range of negative control group T and medicated group S. In agreement to the present study, findings of Biu *et al.*, (2006) ,Patra *et al.,* (2010) who reported significantly reduction in ALP level in the affected chicks. In contrary, Dkhil *et al.,* (2012) and El-Maksoud *et al*., (2014) reported increased ALP level. Decreased alkaline phosphatase activity is due to damage to the bone marrow.

The level of creatinine recorded normal in groups C, D, K ,L, M , N, treated with crude extracts and fractions different concentrations were recorded normal and lies in the close range of negative control group T and medicated group S. while in remaining groups the creatinine level increased abnormally from normal range due to severe infection in these groups. Similarly results of Youssef *et al*., (2013) showed a significant increase of creatinine level in infected chicks. While in contrast work of Biu *et al.,*(2006),Patra *et al.,* (2010), Mondal *et al.,* (2011), El-Maksoud *et al.,* (2014) proposed that there will be significant decrease in creatinine level in coccidia infected chicks.

The normal level of uric acid was recorded in groups C, D, K , L, M, N treated with crud extracts and fractions different concentrations of *C.fistula* and in medicated group S. While a significant increase in uric acid concentration was observed in infected non-medicated positive control group U and in some other groups when compared with uninfected negative control group T and best treated groups. The results

of this study are similar to findings of Biu *et al*., (2006) and Patra *et al.,* (2010) who reported increase in uric acid level in infected chicks. In chicks, catabolism of nitrogen results in a major product the uric acid (Youssef *et al*., 2013). In infected broilers the higher level of uric acid might be due to kidney dysfunction.

Among all groups intestinal digesta viscosity was higher in chicks of uninfected negative control group T followed by group M treated with 10% of fraction 22 of *C. fistula* extract and group N treated with 20% of fraction 22 of *c. fistula* extract. The lowest intestinal digesta viscosity was observed in positive control group U, followed by group A treated with 10% of *F. racemosa* crude extract, group B treated with 20% of *F. racemosa* crude extract. From the results of the present study it is cleared that in *Eimeria* infected groups the intestinal digesta viscosity decrease while in best treated groups the intestinal digesta viscosity is higher. The reduction in intestinal digesta viscosity due to Coccidial infection might be due to osmotic and absorptive changes in the intestinal tract caused by the parasites.

Similar to our findings in a study conducted by Waldenstedt *et al*., (2000) also observed that due to *Eimeria* infection in chicks the intestinal digesta viscosity decreases as compared to healthy and treated groups as they having very high intestinal digesta viscosity.

In present study due to *Eimeria* infection a significant decrease in RBCS and haemoglobin level occur in positive control group U , E treated with 10% *S. cumini* and F treated with 20% *S. cumini* crude extracts. While the best experimental groups showed similar haemoglobin level and RBCs no to the negative control group T normal range of haemoglobin treated with 10% of *C. fistula*, D treated with 20% of *C. fistula*, K treated with 10% of *C.fistula* F-20, L treated with 20% of *C.fistula* F-20, M treated with 10% of *C.fistula* F-22, N treated with 20% of *C. fistula* F-22. Similar to the present study Gotep *et al*., (2016) also observed increase in RBC and haemoglobin concentration in best treated groups and proposed that this is the indicative of the erythropoietic ability of the combined extracts, which is beneficial in the *Eimeria* parasite in the epithelia of the intestines causes bloody diarrhea and consequently anemia. This finding is in consonance with that of Sanni *et al*., (2005) who reported an antianaemic effect of *Khaya senegalensis* on phenylhydrazine-induced anemia in rats. Normal range of WBCs observed in best treated groups C, D, K, L, M, N, medicated

group S and in negative control group T. Increase in total WBCs number and decrease in lymphocytes was observed in positive control group U and in some other crude extracts and fractions treated groups. In best treated groups the dose dependent decrease in white blood cells count and heterophil-lymphocyte ratio with a concomitant reduction in heterophils is suggestive of decreased inflammation. It can be extrapolated that the decrease in parasitic load down regulates the activity of the immune system leading to decrease in inflammation and consequently a decrease in heterophils, tending towards the normal blood picture of a greater ratio of lymphocytes to heterophils in avian species (Ishaq *et al*., 2014; Gotep *et al*., 2016).

Further hematological analysis revealed significantly lower ($P < 0.05$) levels of MCV (fL), MCH (pg) and MCHC (g/l) for chickens in positive control group U and in some other infected groups chicks compared with those levels in non-infected control chickens. The levels of these hematological parameters in chickens infected with *Eimeria* species were also below the normal ranges, whereas the levels in non-infected control chickens and in best treated groups were within the normal ranges. In contrast a study conducted by Kockaya and Ozsensoy, (2016) proposed that the level of MCV were increased significantly in coccidiosis affected lambs. The levels of these hematological parameters like MCV, MCH, MCHc in chickens infected with *Eimeria* species were below the normal ranges, which are consistent with those reported previously (Weiss and Wardrop 2010; Akhtar *et al*., 2015).

For knowing the overall sensitivity/resistance of *Eimeria* species to selected plants extracts, different fractions and commonly used anticoccidial drug amprolium three indexes are used i.e., Anticoccidial index (ACI), anticoccidial sensitivity test (AST), Percent Optimum Anticoccidial Activity (POAA).in present study *Eimeria* species show no resistance and were completely killed by the applied extract of selected plant and their fractions due to their high anticoccidial actions in some groups like c, d, K-N treated with different concentrations of crude extract of selected plants *i.e*., *C. fistula* and their fractions. While the groups i.e A treated with crude extract of *F.racemosa*, group D treated with Salinomycin and group U infected unmedicated showed overall strong resistance. Similarly a study conducted in China where severe resistance to Amprolium hydrochloride, Toltrazuril and Sulfaquinoxaline sodium was reported by Lan *et al*., (2017). In contrast a study conducted by Ojimelukwe *et al*.,

(2018) by adopting these three anticoccidial efficacy indexes ACI, AST and the POAA showed sensitivity to Amprolium.

On 36th day of experiment the mean internal organ weight relative to body weight was remain normal in some best treated groups like C, D, K, L, M, N and in healthy negative control group T. While increased in weight of liver and intestine recorded in some infected groups like positive control group U, E-G due to hypertrophy.

Similarly, a study conducted by Abbas *et al*., (2019) proposed that *Trachyspermum ammi* extract reduce the severity of *Eimeria* infection and had better effect on organ weight of *Eimeria* infected chicks treated with *T. ammi* extract. Awais *et al*., (2014) also find a similar effect on organ weight improvement of coccidia infected chicks with *Saccharum officinarum* extract.

On postmortem observation of the visceral organs in some infected groups. In present study increase in liver, intestine, spleen, discoloration of liver and increase in watery ingesta with mucous and blood was observed. The post-mortem revealed extremely ballooned intestine and hemorrhages were seen while looking grossly without opening the gut. Intestine was often found edematous, thickened, showing necrosis and sloughing of intestinal epithelium and friable. The findings thus recorded coincide with those of Sharma *et al*., (2015).

The normal, healthy and best treated groups like C, D, K, L, M, N, and group T showed normal histological structure of mucosa, sub mucosa and muscularis lamina propria. The caecum of infected chicks after postmortem examination on 36th day of experiment showed epithelial necrosis, presence of vacuolated and degenerated schizonts, desquamation of enterocytes, MNC infiltration and fibrosis in muscularis mucosa was also observed. Massive congestion of submucosal blood vessels. These finding are similar to those of (Zulpo *et al*., 2007; Zhang *et al*., 2012 and Abdel-Wasae and Alashwal, 2014).

Similar to the findings of Zhang *et al*., (2012) and Badria *et al*., (2017) in the present study the normal, healthy and best treated groups like C, D, K, L, M, N, was treated with *C. fistula* crude extracts and their fractions different concentrations showed an effect on several stages and sporulated oocysts of *E .tenella*

indicates that *C. fistula* is able to kill or inhibit the growth and development of oocysts and damaged cecal tissues to recovery after damage in lamina propria and crypts glands and the decrease in inflammatory cells until they have finally disappeared completely.

On histopathological observations of liver, the uninfected group T, some other best treated groups like C, D, K, L, M, N possess normal histology while in other experimental groups Some major pathological changes in liver observed like hepatic congestion, Bi nucleated Hepatocytes also minimal necrosis of hepatocytes with infiltration, Pyknosis, Substantial increase in normal hepatocytes size was observed. Similarly, Mikail *et al*., (2019) also observed the similar histopathological changes in liver of coccidia infected chicks but not best treated groups like A, B, O, P, Q, R, S, U.

In best treated groups the intestine histology is normal while in some other groups which is not treated well by the extracts of selected plants in their intestine Duodenal glands having some parasitic stages of *Eimeria*. Hyperplasia of goblet cells, Several oocysts with in lumen, Disintegration of epithelial cells, Presence of intracellular developmental stages of parasite, hyperplasia of enterocytes due to second generation schizonts and gamonts. Similar histopathological changes observed in the intestine of brown kiwi having enteric coccidiosis reported by Morgan *et al*., (2012). Similarly, Erdogmus *et al*., (2019) also observed similar pathological changes in intestine of *Eimeria* infected chicks in all groups except healthy and probiotic administered groups.

Lesions and tthickening of glomerular basement membrane in the kidneys of chicken infected with *Eimeria* 'U' and in groups not treated well like groups A, B, O, P, Q, R and S. Increased in Bowman s' Space occur in group U, A, B,E-J, O-R. While normal bowman s'space is present in group C,D, K-N, S and T. Renal corpuscular area reduce in size in group A, B, E,F, Q-R, and in group U. While remain normal in group C, D,K-N,and in S,T groups. In the infected untreated chick's mild renal congestion was observed. No lesions were seen in the kidneys of chicks infected and treated with amprolium and different concentrations of the crude methanolic extract of selected plants and their anticoccidial fractions (Mikail *et al*., 2019).

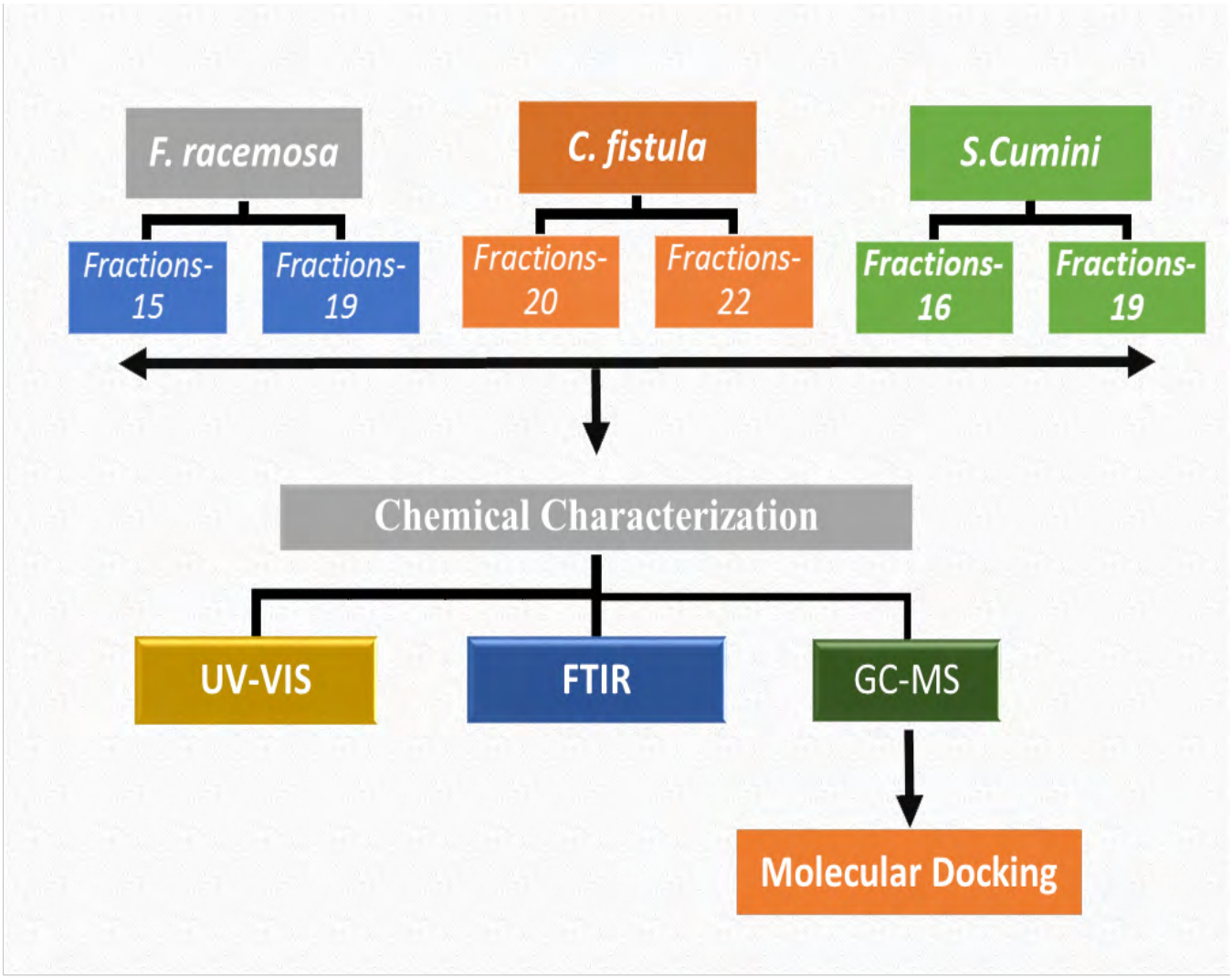
Conclusion

According to the present findings all of the fractions treated groups showed maximum weight gain, feed consumption, best FCR and *etc* as like control and amprolium treated groups. Large scale studies on identification and isolation of bioactive compounds from anticoccidial fractions of the investigated plants are suggested.

Chapter 5

Title: Phytochemical characterization of anticoccidial fractions and in silico molecular docking of identified compounds of experimental plants

Graphical Abstract



ABSTRACT

The anticoccidial active fractions of methanolic extracts of each selected plant species were subjected to phytochemical characterization. Phytochemical characterization by different spectroscopic techniques provides considerable knowledge about the extent and nature of the chemical substances present in extracts. The most effective fractions of *F. racemosa*, *C. fistula* and *S. cumini* were characterized by UV-Vis spectroscopy, FTIR and GC-MS analysis. The identified compounds of each selected fraction were docked with target S-Adenosylmethionine synthase by in silico molecular docking techniques. The UV-vis spectroscopic analysis of anticoccidial fractions was performed at 200 to 800nm and different absorption peaks were recorded. The FTIR analysis was performed for the identification of different functional groups based on characteristic peak values and the spectrum was recorded by an FTIR spectrometer with wavelength scan range of 400 to 4000 cm^{-1}. The FTIR analysis of selected fractions of each experimental plant confirmed the presence of amino acid, alkanes, alkynes, amines, aldehydes, carboxylic acid and etc. Their GC-MS analysis revealed the presence of some common chemical compounds including Oleic Acid, n-Hexadecanoic acid, Octadecanoic acid, Trichloromethane etc. Among all docked compounds cyclododecane methanol of F-19 and oleic acid of F-15 of *F. racemosa* have highest binding affinity with target S-Adenosylmethionine synthase. These compounds are ideal for the synthesis of plants derived anticoccidial medicines.

INTRODUCTION

In providing rural residents with basic healthcare, plants are important. They act as both therapeutic agents and significant raw materials in the creation of both natural and synthetic medicines. Exporting medicinal plants to other nations can generate a sizeable sum of foreign currency. In this sense, local medicinal plants contribute significantly to a nation's economy. For millennia, numerous traditional medical systems have used plant extracts and other forms of plant to cure human illnesses, especially those brought on by pathogenic bacteria, fungus, and viruses (Ray *et al*., 2004; Verma *et al*., 2010). Plants are utilized in the pharmaceutical industry as insecticides, flavoring agents, colours, and other fascinating biological functions. (Jayapriya and Shoba, 2015). Phytochemical screening is a useful approach for finding chemicals in plant extracts that may have use in ethno pharmacology. Various secondary metabolites extracted from crude plant extract have considerably different chemical properties depending on the species. Since from the start phytochemicals produced from plants have been used in medicine. These substances can be obtained from different parts of plant like leaves, flowers, roots, seeds, bark, and stems. (Yadav and agarwala, 2011).

A useful method for the qualitative examination of many secondary metabolites is phytochemical characterization (UV-Vis, FTIR, and GC-Ms). When molecules, atoms, or ions of a compound are exposed to electromagnetic radiation in the ultraviolet and visible ranges, they either absorb it or emit it. This process is known as spectroscopy. Sample transitions from one energy state to another. UV spectroscopy is type of absorption spectroscopy in which light of ultra-violet region (200-400 nm) is absorbed by the molecule which results in the excitation of the electrons from the ground state to higher energy state. Basically, spectroscopy is concerned with how light interacts with matter. The energy content of the atoms or molecules increases as a result of matter absorbing light. When UV rays are absorbed, this causes the electrons to be excited out of the ground state and into a higher energy state. In order to excite their electrons to higher anti-bonding molecular orbitals, molecules with -electrons or non-bonding electrons (n-electrons) can absorb energy in the form of UV light (Kumar, 2008). A chemical molecule's ability to absorb UV light results in a specific spectrum

that makes it easier to identify the component. It is useful in the structure elucidation of organic molecules, such as in detecting the presence or absence of unsaturation, the presence of hetero atoms.

UV absorption spectroscopy can characterize those types of compounds which absorbs UV radiation thus used in qualitative determination of compounds. Identification is done by comparing the absorption spectrum with the spectra of known compounds. This technique is used to detect the presence or absence of functional group in the compound. Absence of a band at particular wavelength regarded as an evidence for absence of particular group. UV spectrophotometer may be used as a detector for HPLC (Aryal, 2018). An analytical method that makes use of a molecule's vibrational transitions is infrared (IR) or vibrational spectroscopy. Due to its utility in identifying compounds and establishing their structures, it is one of the most popular and commonly utilized spectroscopic methods used mostly by inorganic and organic chemists. In order to create an infrared spectrum, the infrared spectrometer (also known as a spectrophotometer) is used in conjunction with the infrared spectroscopy method or methodology. Infrared spectrometers allow chemists to produce absorption spectra of compounds that are a distinctive reflection of their molecular structure. These spectra are similar in concept to other spectrometers. An infrared spectrum, which is a plot of measured infrared intensity vs wavelength (or frequency) of light, is the primary measurement acquired by infrared spectroscopy. Based on the measurements of atom vibrations made by IR Spectroscopy, the functional groups of molecules can be identified (Stuart, 1996).

The general rule is that heavier atoms and stronger bonds will vibrate with a higher stretching frequency. For scientific researchers, it has been very important in a variety of domains, including the examination of gaseous, liquid, or solid materials, the identification of substances, and quantitative analysis. The IR spectrum may be used to infer details about the structure and functional groups of molecules. It can also be used to learn how molecules interact (Aryal, 2021). An analytical technique combines the advantages of gas chromatography and mass spectrometry (GC-MS). The sample either evaporates as a gas or a liquid in the injection port. In gas chromatography, helium is widely employed as the mobile phase due to its low molecular weight and chemical

inertness. The mobile phase pulls the analyte through the column when pressure is applied. Utilizing a column coated with a stationary phase, the separation is carried out. GC is frequently employed in situations involving non-aqueous fluids and the detection of tiny, volatile compounds. In GC, non-polar compounds are preferred (Aryal, 2020). Therefore, GC-MS is a combination of the two analytical techniques used to analyze complex biochemical and organic molecules (Skoog *et al*., 2017). By using a mass spectrometer, the spectrum of isolated compounds is recorded, and the compounds are characterized based on their mass to charge ratio (Hussain and Maqbool, 2014). Anticoccidial active fractions identified compounds interaction with targets S-Adenosylmethionine synthase were studied by molecular docking simulation. S-Adenosylmethionine synthase is crucial for the life of the *Eimeria* species as it is responsible for cell proliferation, synthesis of polyamines and gene transcription (Maheswari and Revathi., 2016).

Phytochemical extraction and characterization from the green plant have opened a new gateway to some high activity profile drugs. The synthesis of complex chemical compounds from these bioactive substances requires more precise knowledge about their chemical constituents (Vaghasiya *et al*., 2011). In present study, phytochemical analysis and chemical characterization of anticoccidial active fractions of experimental plants and in silico molecular docking of identified compounds were carried out for the identification of best docked compounds.

MATERIALS AND METHODS

Chemical characterization of anticoccidial active fractions 15 and 19 of *F. racemosa*, fraction 20 and 22 of *C. fistula* and fraction 16 and 19 of *S. cumini* were carried out by UV-Vis spectroscopy, FTIR and GC-MS analysis for the identification of compounds.

Ultra violet visible spectroscopy (UV-Vis)

UV-visible spectrophotometric analysis was conducted for spectral profiling of active fractions of selected plants by using a UV-visible spectrophotometer (UV-1601, Shimadzu). The samples were centrifuged for 10 minutes at 3000 rpm and filtered by using Whatman No.1 filter paper. Samples 10 times dilution was done with mother solvent methanol. Characteristics peaks of UV-VIS were recorded after scanning of diluted samples at wavelengths ranging approximately from 200 to 800 nm (Dhivya and Kalaichelvi., 2017; Tripathy *et al*., 2016).

Fourier transform infrared spectroscopy (FTIR)

For the identification of functional groups in active fractions, Fourier transform infrared spectroscopy (FTIR) was used. The samples for FTIR were prepared by mixing a small quantity (1mg) of each active fraction powder with 10mg of potassium bromide (KBr). After mixing and compressing, thin discs were prepared which were then loaded in FTIR spectrometer (Shimadzu, Japan) with wavelength scan range of 400 to 4000 cm^{-1}. Characteristics peak values were recorded (Kalaichelvi and Dhivya., 2017; Sahu and Saxena, 2014).

Gas chromatography and Mass spectroscopy (GC-MS)

The active fractions 15 and 19 of *F. racemosa* leaves methanolic extract were subjected to Gas chromatography and mass spectrometry. HP-5MS capillary column (30 m x 0.25 mm x 0.25µm film thickness) was used. The temperature of the oven was kept at 80°C to 300°C. Nitrogen (99.99%) was used as carrier gas with an injection volume of 1.0 μl and at a flow rate of 1.0 ml/min was employed. Operation at an electron energy of 70 eV of electron-impact ionization of the mass spectrometry was done. For each fraction, the total GC running time was 20 minutes. The retention time of each

spectrum of chromatogram was compared with the NIST library for the identification of corresponding compounds, their structure, molecular formula, and weight (Sivakumar *et al*., 2019).

Identification of Phytoconstituents

National institute of standard and technology (NIST) database having more than 62000 patterns was used for the interpretation of mass spectrum. The comparison between spectra of known and unknown compounds from the NIST library, help in the interpretation of the mass spectrum and the determination of names, chemical formula, chemical structure and molecular weight of compounds in different anticoccidial active fractions of selected plants (Rukhsana *et al*., 2017).

Molecular Docking

Targets

To determine the anticoccidial potential of identified bioactive phyto compounds of active fractions *i.e*., Cyclodecane methanol and Oleic acid, the three-dimensional structure of target enzyme S-adenosyl methionine synthase that play important role in conversion of intermediate stages of the life cycle of *Eimeria* species, was chosen for docking study and their 3D structure were retrieved from the PDB database (Rathinavel *et al*., 2019).

Generation of Ligands

Cyclodecane methanol and oleic acid served as ligand and SMILES notation of their 2D structures was obtained. The SMILES notation was converted into 3D SDF format by submitting it to online SMILES convertor and structure file generator.

Interaction of protein-Ligands by AutoDock

AutoDock Vina was used for automated flexible docking of ligands for obtaining the binding affinities between target S-Adenosyl methionine synthase and bioactive compounds Cyclodecane methanol and oleic acid from active fractions. The protein binding sites for docking were assessed from a grid map with the help of AutoGrid (Trott and Olson., 2010).

RESULTS

Ultraviolet- Visible Spectroscopic Analysis (UV-Vis)

Qualitative UV-Vis spectroscopic analysis of active fractions of selected plants showed a spectrum profile at 200 to 800nm due to sharpness of peaks and proper baseline. The peak spectra of fraction 15 of *F. racemosa* leaves extract showed characteristic peaks at 207, 226, 229, 231, 338, 441, 552, 555 and 660nm with the absorption of 2.3, 1.2, 1.3, 0.7, 1.4, 0.2,0.1,0.3 and 0.4 respectively. The observed peaks of fraction 19 of *F. racemosa* were at 202, 210, 332, 429, 470, 537 and 564nm with absorption of 1.6, 1.2,0.6, 0.2, 0.1, 0.1 and 0.1 respectively. Anticoccidial active fraction 20 of *C. fistula* leaves extract showed characteristic peaks at 207, 240, 283, 295, 370 and 400nm having absorbance of 2.0, 1.1, 1.2, 0.6, 0.5 and 0.2 respectively. Peaks of fraction 22 of *C. fistula* were at 240, 254, 290, 340, 400, 420 and 665nm with absorption of 2.0, 1.8, 1.1, 1.2, 0.3, 0.2 and 0.1 respectively.

S. cumini leaves extract active fraction 16 peaks were at 220, 243, 249, 332 and 390nm with absorption spectra of 1.6, 0.7, 0.6 0.3 and 0.1 respectively while fraction 19 peaks were at 220, 240, 256, 310 and 335nm with the absorption of 2.0, 0.9, 0.8, 0.3 and 0.1 respectively (Table 5.1-5.6 and Figure 5.1 -5.6).

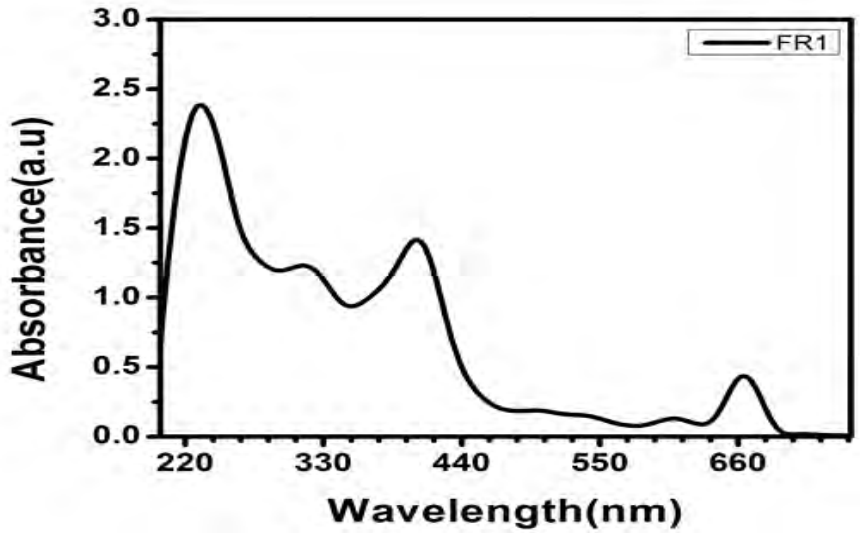


Figure 5.1: UV-Vis spectra of fraction 15 of *F.racemosa.*

Table 5.1: UV-Vis spectrum values of fraction 15 of *F.rcemosa.*

S.N	Wavelength (nm)	Absorbance
1	207	2.3
2	226	1.2
3	229	1.3
4	231	0.7
5	338	1.4
6	441	0.2
7	552	0.1
8	555	0.3
9	660	0.4

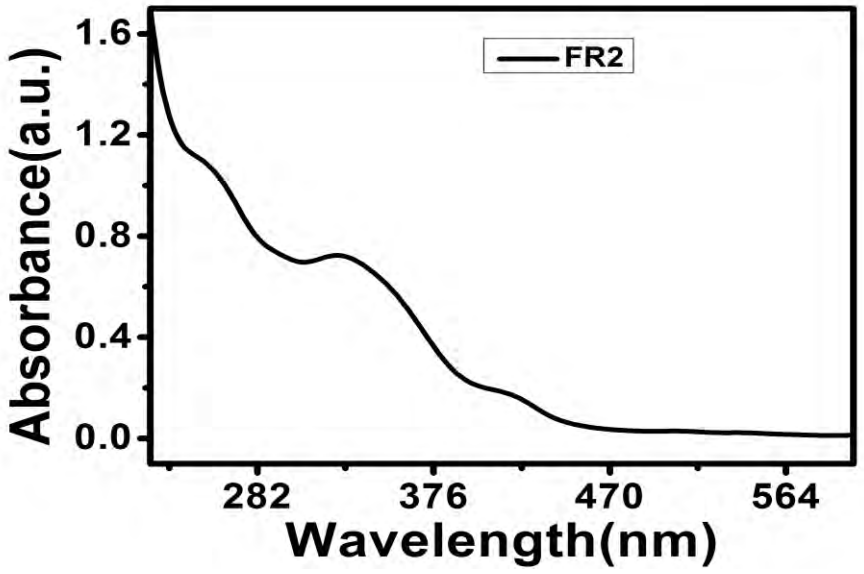


Figure 5.2: UV-Vis spectra of fraction 19 of *F.rcemosa.*

Table 5.2: UV-Vis spectrum values of fraction 19 of *F.rcemosa*

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S.N	Wavelength nm.	Absorbance
1	202	1.6
2	210	1.2
3	332	0.6
4	429	0.2
5	470	0.1
6	537	0.1
7	564	0.1

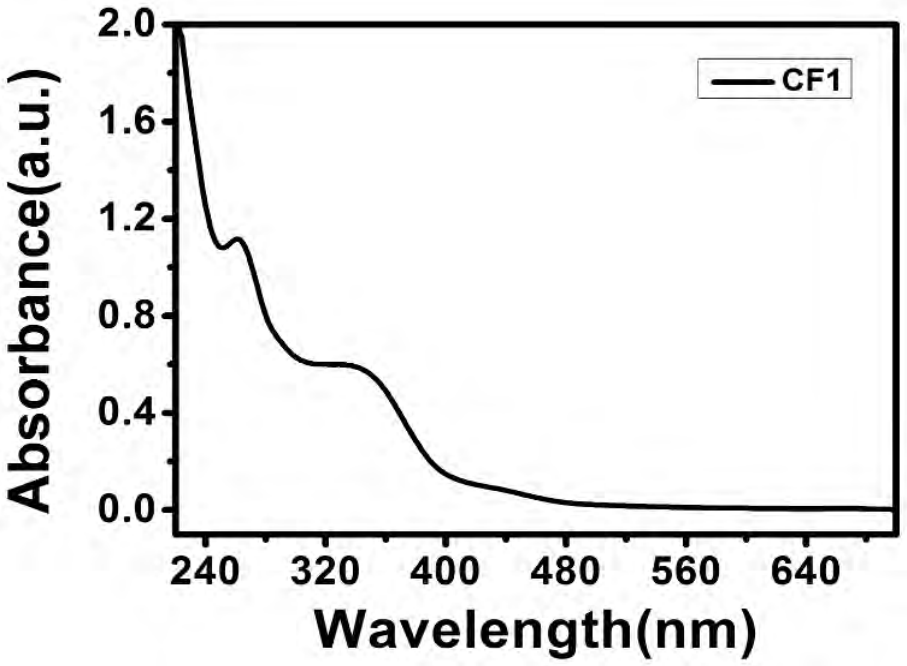


Figure 5.3: UV-Vis spectra of fraction 20 of *C. fistula.*

Table 5.3: UV-Vis spectrum values of fraction 20 of *C.fistula*

S.N	Wavelength nm.	Absorbance
1	207	2.0
2	240	1.1
3	283	1.2
4	295	0.6
5	370	0.5
6	400	0.2

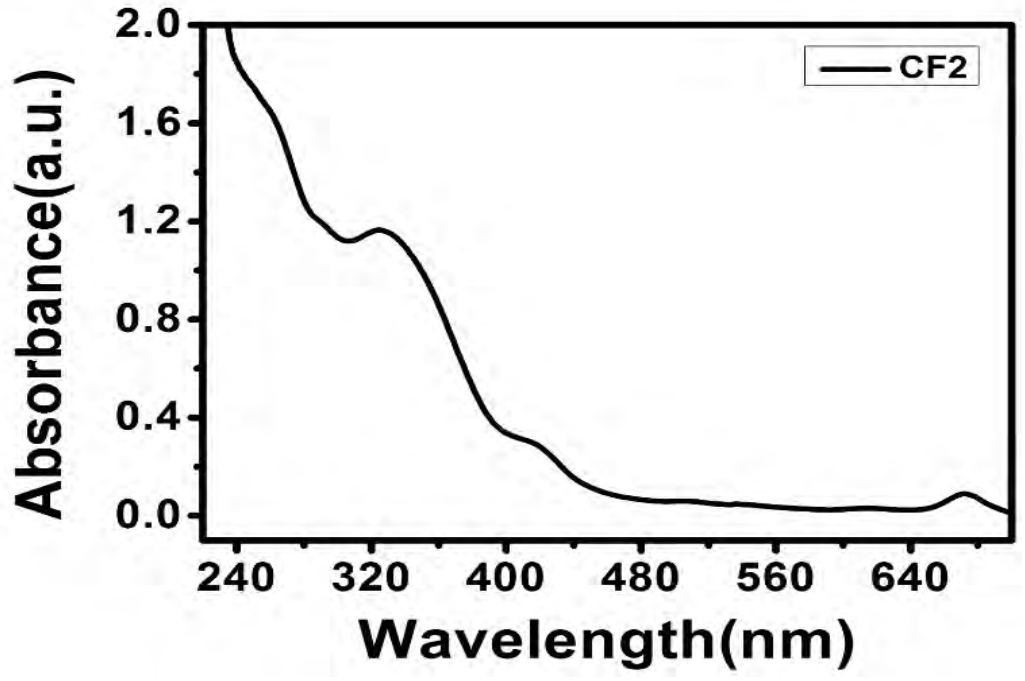


Figure 5.4: UV-Vis spectra of fraction 22 of *C. fistula.*

Table 5.4: UV-Vis spectrum values of fraction 22 of *C.fistula.*

S.N	Wavelength nm.	Absorbance
1	240	2.0
2	254	1.8
3	290	1.1
4	340	1.2
5	400	0.3
6	420	0.2
7	665	0.1

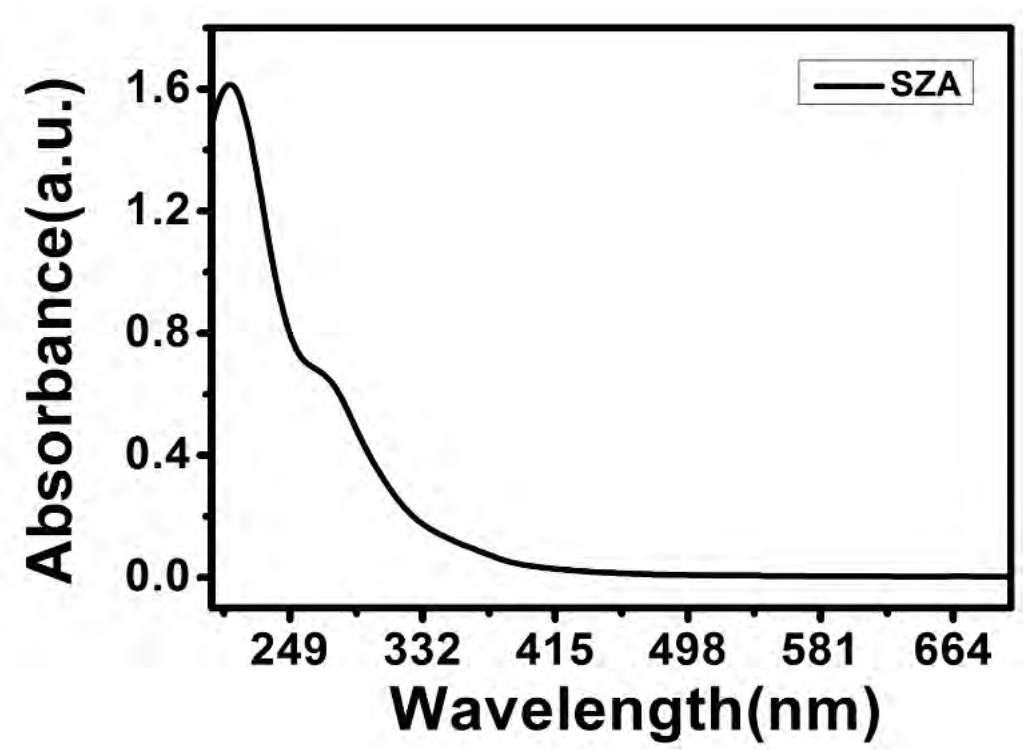


Figure 5.5: UV-Vis spectra of fraction 16 of *S.cumini.*

Table 5.5: UV-Vis spectrum values of fraction 16 of *S.cumini.*

S.N	Wavelength nm.	Absorbance
1	220	1.6
2	243	0.7
3	249	0.6
4	332	0.3
5	390	0.1

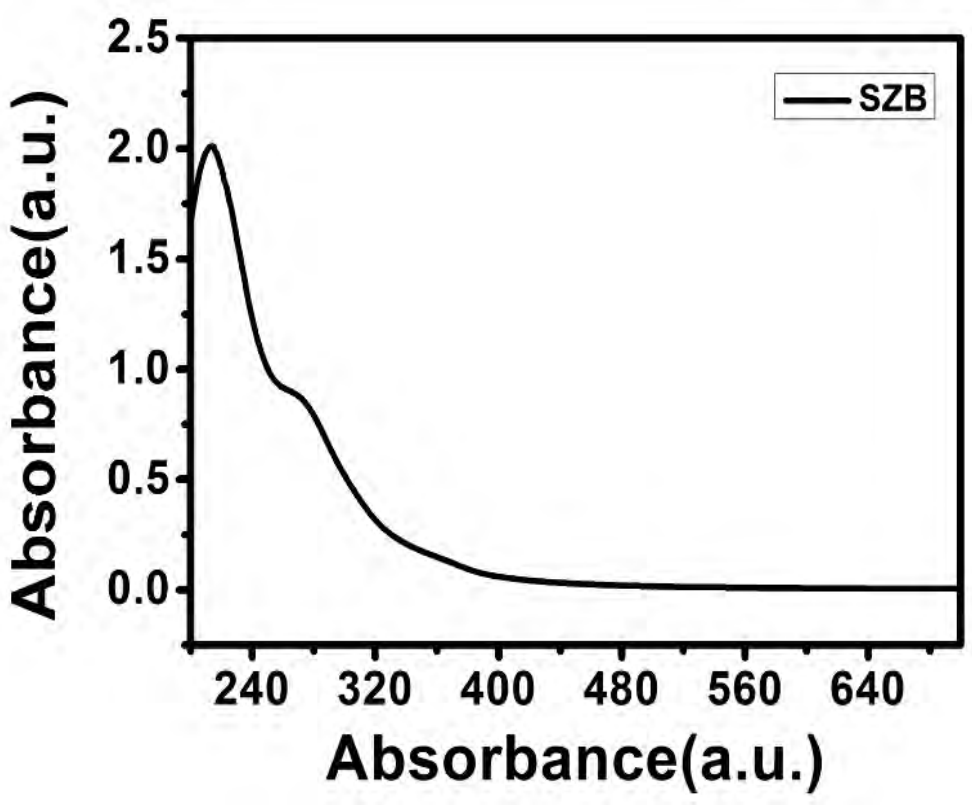


Figure 5.6: UV-Vis spectra of fraction 19 of *S.cumini.*

Table 5.6: UV-Vis spectrum values of fraction 19 of *S.cumini*

S.N	Wavelength nm.	Absorbance
1	220	2.0
2	240	0.9
3	256	0.8
4	310	0.3
5	335	0.1

Fourier transform infrared Spectroscopy (FTIR)

The FTIR analysis of the anticoccidial active fractions of selected plants leaves extract helps in the identification of functional groups of active compounds based on peak values present in the region of infrared radiation. Each peak in spectrum represent the specific value corresponding to specific functional groups. The absorbance was measured from 400 to 4000cm^{-1} wavelength. In present study the results of FTIR analysis of the active fractions confirmed the presence of amines, alkanes, alkynes, alkene, phenols, aldehydes, Carboxylic Acid, Imine, halo compounds etc.

The FTIR spectrum of the fraction 15 of the *F. racemosa* leaves extract gave broad peak at 3371.51cm^{-1} which indicate the presence of N-H stretching correspond to alcohol/Amine. The peaks obtained at 2955.59 and 2924.00cm^{-1} indicate the presence of O-H stretching of Carboxylic Acid. While the presence of amine salt was confirmed from the peak value 2854.03 cm^{-1} with N-H stretching. The peaks obtained at 1712.78 and 1626.01cm^{-1} indicate the presence of C=O and C=C stretching of carboxylic acid and alkene respectively. The peaks obtained at 754.79 and 608.59 cm^{-1} indicate the presence of C-Cl stretching of halo compounds (Figure 5.7: Table 5.7).

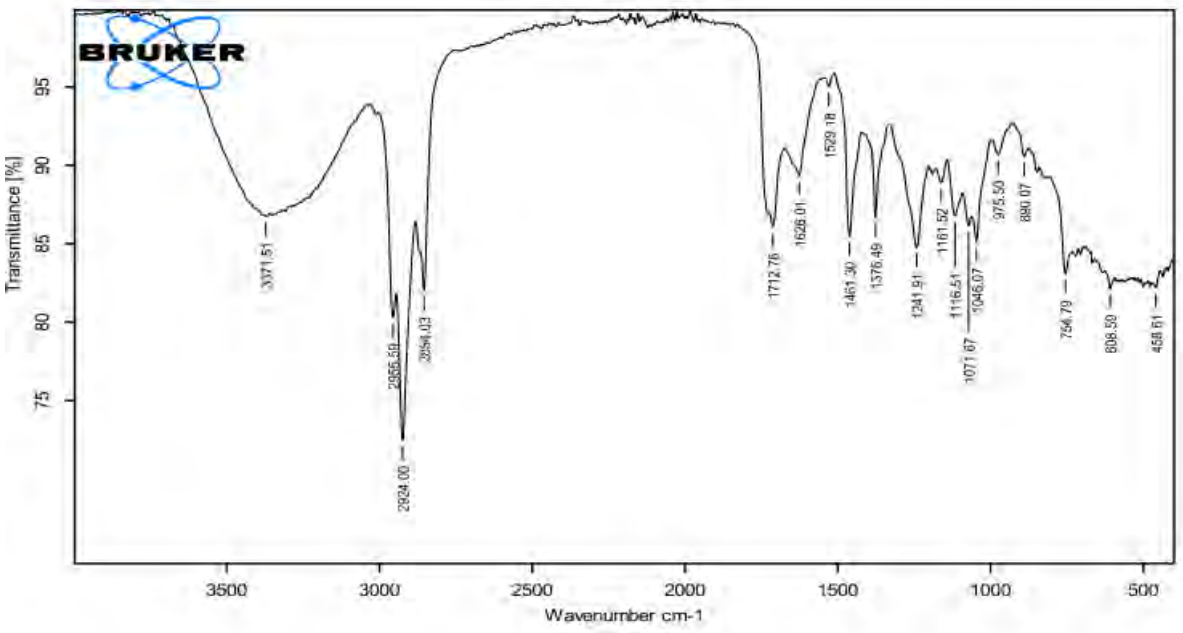


Figure 5.7: FTIR spectrum of fraction 15 of *F.racemosa* leaves extract

Table 5.7: FTIR spectral peak values and functional groups of fraction 15 of *F.racemosa* leaves extract.

Peak No	Peak Value	Type of Stretching	Functional Group
1	3371.51	O-H / N-H stretching	Alcohol/Amine
2	2955.59	O-H stretching	Carboxylic Acid
3	2924.00	O-H stretching	Carboxylic Acid
4	2854.03	N-H stretching	Amine Salt
5	1712.78	C=O stretching	Carboxylic Acid
6	1626.01	C=C stretching	Alkene
7	1529.18	N-O stretching	Nitro Compounds
8	1461.30	C-H bending	Alkane
9	1376.49	O-H bending	Phenols
10	1241.91	C-O stretching	Alkyl Aryl Ether
11	1161.52	C-N stretching	Amine
12	1116.51	C-O stretching	Aliphatic Ether
13	1071.67	C-O stretching	Primary Alcohol
14	1046.07	S=O/ CO-O-CO stretching	Sulfoxide/Anhydride

15	975.50	C=C bending	Alkene
16	890.07	C=C bending	Alkene
17	754.79	C-Cl stretching	Halo Compounds
18	608.59	C-Cl stretching	Halo Compounds
19	458.61	C-I stretching	Halo Compounds

Anticoccidial fraction 19 of *F. racemosa* leaves extract gave a strong peak at 3329.03, 2974.47 and2928.65 cm^{-1} indicate the presence of O-H stretching of carboxylic acid. The peak obtained at 2895.74 cm^{-1} indicate the presence of N-H stretching of amine salt. The presence of imine / Oxime stretching was confirmed from the peak value 1649.56 cm^{-1} with C=N stretching. The C-H bending of alkane was confirmed from the peak value 1451.94 cm^{-1} . the obtained peak value 1326.79 cm^{-1} confirmed the presence of O-H bending of phenols. The obtained other values of the peaks 1274.32 (C-N), 1087.00(C-O), 1044.96 (CO-O-CO), 879.23(C-H), 603.72(C-Cl), 432.17(C-I) confirmed the presence of Aromatic Amine, Secondary Alcohol, Anhydride, 1,2,4-Trisubstituted and Halo Compounds respectively (Figure 5.8 and Table 5.8).

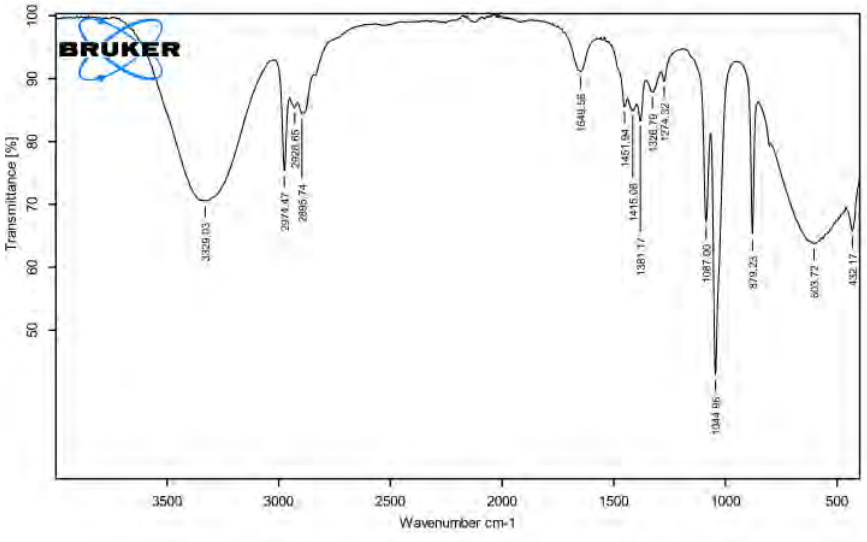


Figure 5.8: FTIR spectrum of fraction 19 of *F.racemosa* leaves extract.

Table 5.8: FTIR spectral peak values and functional groups of fraction 19 of *F.racemosa* leaves extract

Peak No	Peak Value	Functional Group	Functional Group Name	Vibrations	Intensity
1	3329.03	N-H/O-H	Amine/Alcohol	stretching	strong
2	2974.47	O-H	Carboxylic Acid	stretching	strong
3	2928.65	O-H	Carboxylic Acid	stretching	strong
4	2895.74	N-H	Amine Salt	stretching	strong
5	1649.56	C=N	Imine / Oxime	stretching	strong
6	1451.94	C-H	Alkane	bending	weak
7	1415.08	O-H	Alcohol	bending	weak
8	1381.17	S=O	Sulfate	stretching	strong
9	1326.79	O-H	Phenol	bending	weak
10	1274.32	C-N	Aromatic Amine	stretching	strong
11	1087.00	C-O	Secondary Alcohol	stretching	Strong
12	1044.96	CO-O-CO	Anhydride	stretching	Strong
13	879.23	C-H	1,2,4-Trisubstituted	Bending	weak
14	603.72	C-Cl	Halo Compounds	stretching	strong
15	432.17	C-I	Halo Compounds	stretching	strong

In anticoccidial active fraction 20 of *C. fistula* leaves extract, peak values of 3333.61, 2946.23 and 2834.89 cm^{-1} confirmed the presence of N-H / O-H stretching of strong amine salt. Peak values 1650.61 and 1449.55 cm^{-1} indicate the presence of C-H weak and medium bending of alkane respectively. Peak values of 1409.73 cm^{-1} confirmed the presence of S=O stretching of sulfonyl chloride. The peak values 1261.09 and 1113.05 cm^{-1} clearly indicate the presence of C-O strong stretching of aliphatic compounds. The peak value 1017.72 cm^{-1} confirmed the presence of C-F stretching of fluro compounds. The peak values 580.05 and 462.06 cm^{-1} indicate the presence of C-Cl and C-H stretching and bending of halo compounds and 1,2,4-trisubstituted respectively (Figure 5.9 and Table 5.9).

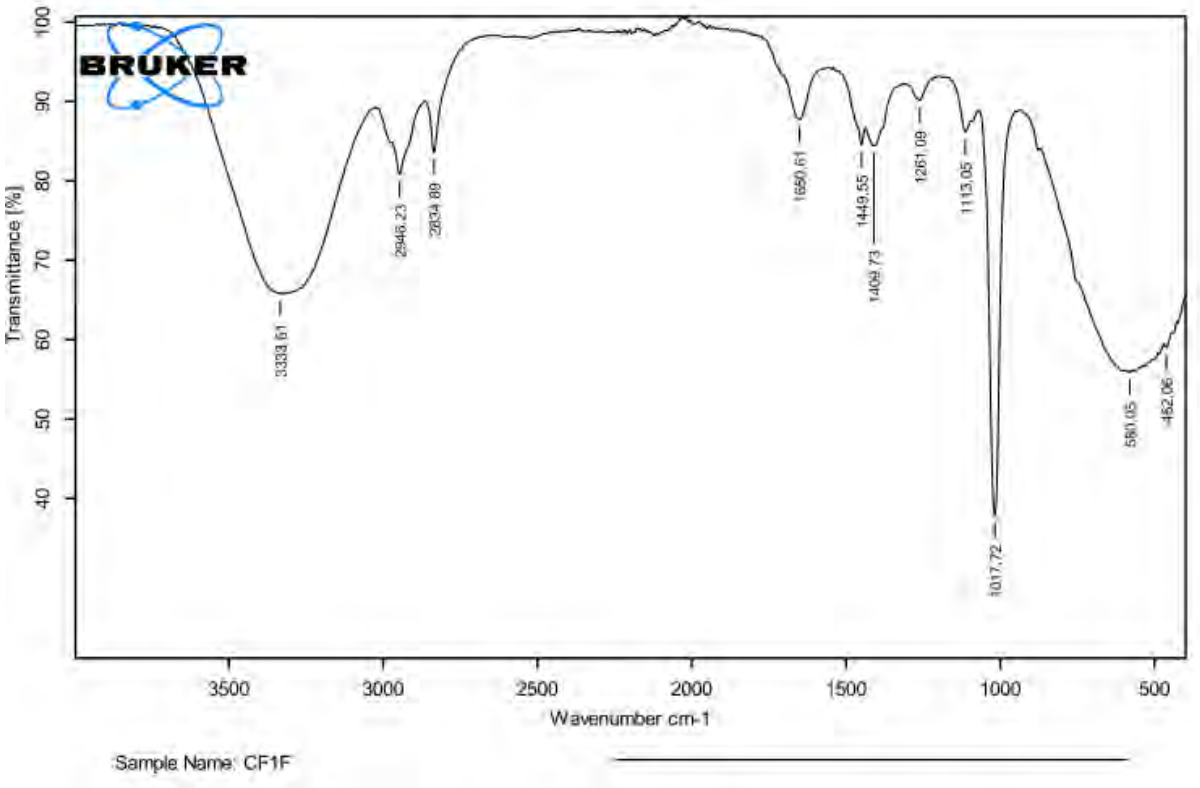


Figure 5.9: FTIR spectrum of fraction 20 of *C.fistula* leaves extract

Table 5.9: FTIR spectral peak values and functional groups of fraction 20 of *C.fistula* leaves extract.

Peak No	Peak Value	Functional Group	Functional Group Name	Vibrations	Intensity
1	3333.61	O-H	Alcohol	stretching	strong
2	2946.23	O-H/ N-H	Alcohol /Amine Salt	stretching	strong
3	2834.89	N-H	Amine Salt	stretching	strong
4	1650.61	C-H	Aromatic Compounds	Bending	Weak
5	1449.55	C-H	Alkane	Bending	Medium
6	1409.73	S=O	sulfonyl chloride /Sulfate	Stretching	Strong
7	1261.09	C-O	aromatic ester	Stretching	Strong
8	1113.05	C-O	aliphatic ether	stretching	Strong
9	1017.72	C-F	fluoro compound	stretching	Strong

10	580.05	C-Cl	halo compound	Stretching	Strong
11	462.06	C-H	1,2,4-trisubstituted	Bending	Strong

C. fistula leaves extract anticoccidial active fraction 22 of, the peak value 3354.49 cm^{-1} indicate the presence of N-H stretching of aliphatic primary amine. The peak value 2976.26 cm^{-1} confirmed the presence of O-H stretching of alcohol. The peak values 2927.52, 2900.49, 1645.65 and 1452.70 cm^{-1} confirmed the presence of alkane, amine salt, imine and aromatic compounds due to presence of their respective stretching i.e. C-H, N-H, C=N, and C=C respectively. The peak values 1414.92, 1380.25, 1326.30, 1272.97, 1086.14, 1044.14, 878.28 and 570.40 cm^{-1} confirmed the presence of Alcohol, Sulfate, Phenol, aromatic ester, aliphatic ether, Anhydride, Alkene and halo compounds due to the presence of their respective O-H, S=O, O-H, C-O, C-O, CO-O-CO, C=C and C-Br respectively (Figure 5.10 and Table 5.10).

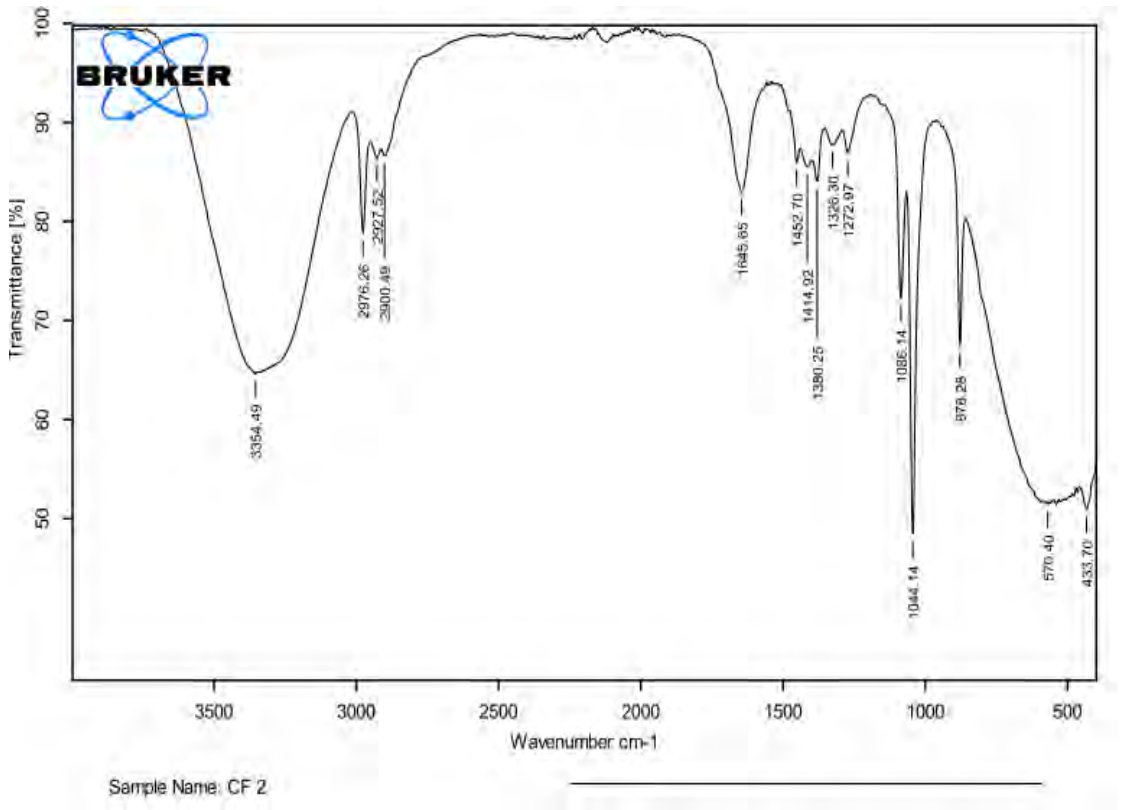


Figure 5.10: FTIR spectrum of fraction 22 of *C. fistula* leaves extract.

Table 5.10: FTIR spectral peak values and functional groups of fraction 22 of *C.fistula* leaves extract.

Peak No	Peak Value	Functional Group	Functional Group Name	Vibrations	Intensity
1	3354.49	N-H	Aliphatic primary amine	Stretching	Medium
2	2976.26	O-H	Alcohol	Stretching	Weak
3	2927.52	C-H	Alkane	stretching	Medium
4	2900.49	N-H	Amine salt	Stretching	Strong
5	1645.65	C=N	imine / oxime	Stretching	Medium
6	1452.70	C=C	Aromatic	Stretching	Weak
7	1414.92	O-H	Alcohol	Bending	Medium
8	1380.25	S=O	Sulfate	Stretching	Strong
9	1326.30	O-H	Phenol	Bending	Medium
10	1272.97	C-O	Aromatic ester	Stretching	Strong
11	1086.14	C-O	Aliphatic ether	Stretching	Strong
12	1044.14	CO-O-CO	Anhydride	stretching	Strong
13	878.28	C=C	Alkene	Bending	Strong
14	570.40	C-Br	Halo compound	Stretching	Strong

In *S. cumini* leaves extract the most active anticoccidial fraction 16 FTIR analysis showed different peaks. The peak value 3340.09, 2945.79, 2834.25, 1650.65 and 1449.46 cm^{-1} confirmed the presence of Alcohol, Alkane, Amine salt, Alkane and Alkyl due to presence of their respective O-H, C-H, N-H, C=C and CH3 respectively. Similarly, the peak values 1411.46, 1259.56, 1112.86, 1018.68, 754.99, 602.56 cm^{-1} confirmed the presence of carboxylic acid, fluoro compound, secondary alcohol, fluoro compound, halo compound and halo compound due to the presence of their respective O-H, C-F, C-O, C-F, C-Cl and C-Br respectively (Figure: 5.11 and Table: 5.11).

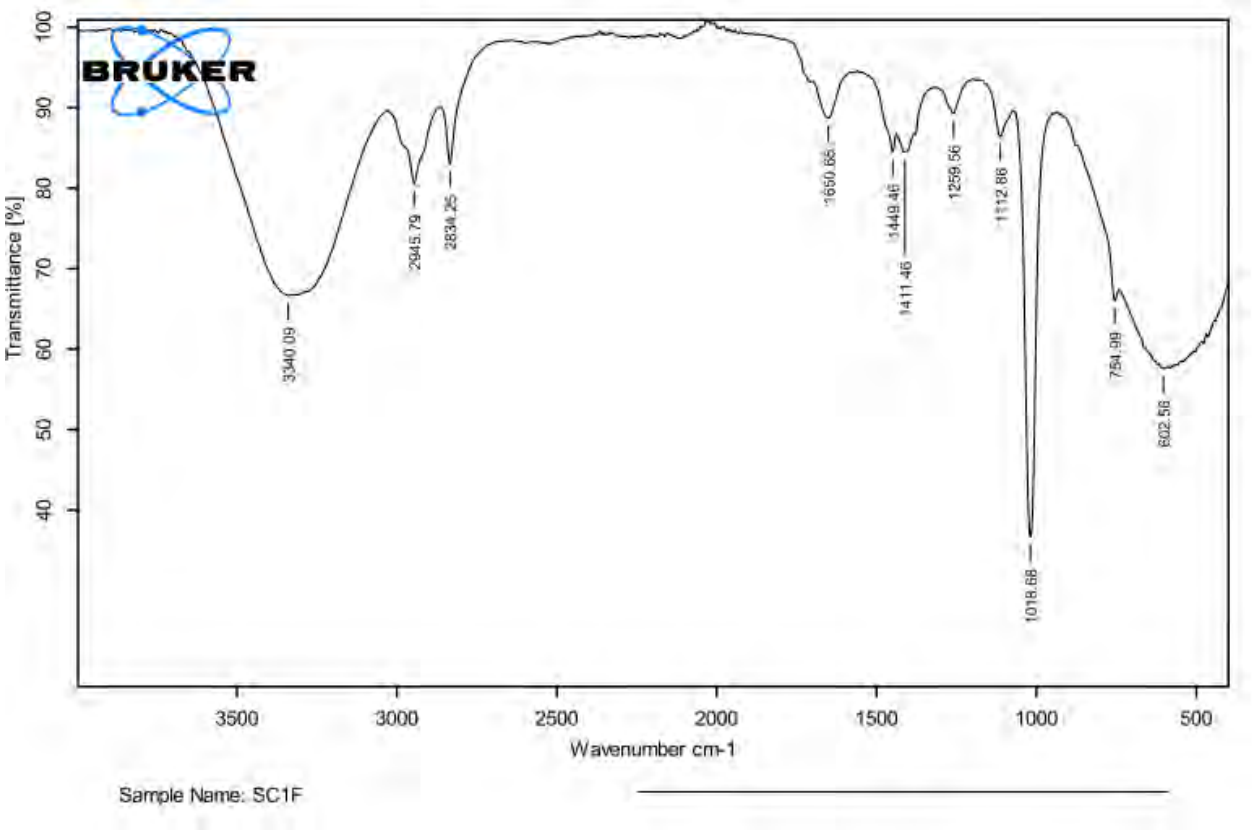


Figure 5.11: FTIR spectrum of fraction 16 of *S. cumini* leaves extract.

Table 5.11: FTIR spectral peak values and functional groups of fraction 16 of *S.cumini* leaves extract.

Peak No	Peak Value	Functional Group	Functional Group Name	Vibrations	Intensity
1	3340.09	O-H	Alcohol	Stretching	Strong
2	2945.79	C-H	Alkane	Stretching	Medium
3	2834.25	N-H	Amine salt	Stretching	Strong
4	1650.65	C=C	Alkane	Stretching	Medium
5	1449.46	CH3	Alkyl	Bending	Medium
6	1411.46	O-H	Carboxylic acid	Bending	Medium
7	1259.56	C-F	Fluoro compound	Stretching	Strong
8	1112.86	C-O	Secondary alcohol	Stretching	Strong
9	1018.68	C-F	Fluoro compound	Stretching	Strong

10	754.99	C-Cl	Halo compound	Stretching	Strong
11	602.56	C-Br	Halo compound	Stretching	Strong

The second most active anticoccidial fraction of *S. cumini* leaves extract is fraction 19. Their FTIR analysis showed different peaks having values 3319.37, 2975.81, 2946.64, 2836.30, 1649.54 and 1450.01 cm^{-1} confirmed the presence of aliphatic primary amine, alcohol, carboxylic acid, amine salt, amine and alkane due to the presence of their respective N-H, O-H, O-H, N-H, N-H and C-H respectively. Similarly, the peaks value 1409.00, 1261.24, 1110.60, 1087.93 and 1017.29 cm^{-1} confirmed the presence of sulfonyl chloride, alkyl aryl ether, aliphatic ether, Secondary alcohol and fluoro compound to the presence of their respective S=O, C-O, C-O, C-O and C-F respectively (Figure 5.12: Table: 5.12).

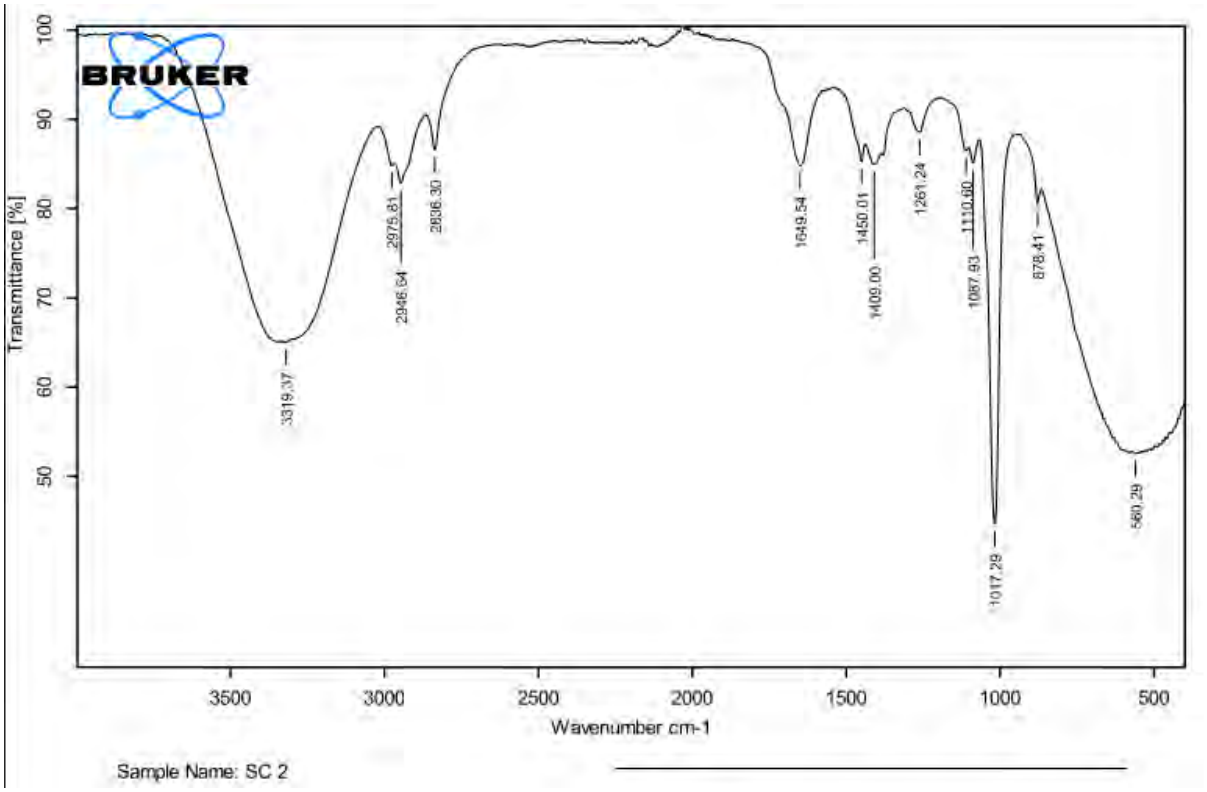


Figure 5.12: FTIR spectrum of fraction 19 of *S. cumini* leaves extract.

Table 5.12: FTIR spectral peak values and functional groups of fraction 19 of S.cumini leaves extract.

Peak No	Peak Value	Functional Group	Functional Group Name	Vibrations	Intensity
1	3319.37	N-H	Aliphatic primary amine	Stretching	Medium
2	2975.81	O-H	Alcohol	Stretching	weak
3	2946.64	O-H	Carboxylic acid	Stretching	Strong
4	2836.30	N-H	Amine salt	Stretching	Strong
5	1649.54	N-H	Amine	Bending	medium
6	1450.01	C-H	Alkane	Bending	Medium
7	1409.00	S=O	Sulfonyl chloride	Stretching	Strong
8	1261.24	C-O	Alkyl aryl ether	Stretching	Strong
9	1110.60	C-O	Aliphatic ether	Stretching	Strong
10	1087.93	C-O	Secondary alcohol	Stretching	Strong
11	1017.29	C-F	Fluoro compound	Stretching	Strong
13	560.29	C-I	Halo compound	Stretching	Strong

Gas Chromatograph Coupled Mass Spectroscopy (GC-MS)

GC-MS analysis of fraction 15 of *F. racemosa*

The GC-MS chromatography of anticoccidial active fraction 15 isolated from methanolic leaves extract of *F. racemosa* showed 5 peaks which confirmed the presence of 5 bioactive compounds. The mass spectrum of each compound was characterized by comparison with the NIST library. Compound names, retention time, area percentage, molecular weight and molecular formula of identified compounds are listed in table 5.13. The major bioactive compounds in fraction 15 are acetone (48.47%), Oleic Acid (37.85%), n-Hexadecanoic acid (7.60%), Octadecanoic acid (5.62%) and Trichloromethane (0.46%) are present in minor quantity. The GC-MS chromatogram of fraction 15 is represented in figure 5.13.

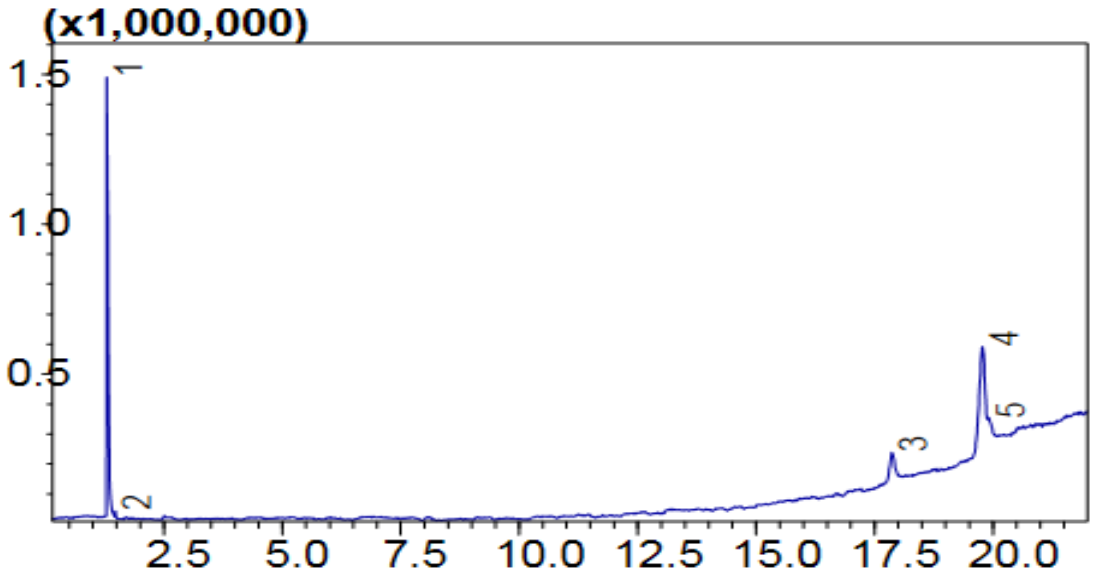
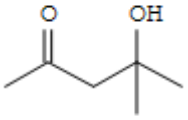
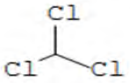
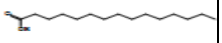
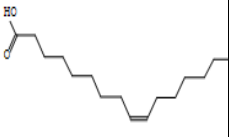
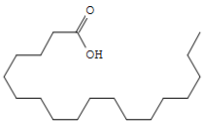


Figure: 5.13: GC-MS chromatogram of fraction 15 of *F. racemosa.*

Table 5.13. Spectral data of GC-MS chromatogram of fraction 15 of *F.racemosa*.

S.N	Peak Retention time	Peak Area (%)	Molecular formula	Molecular weight	Compound analyzed	Compound structure
1	1.296	48.47	C_3H_6O	58	Acetone	
2	1.475	0.46	$CHCl_3$	118	Trichloromethane	
3	17.866	7.60	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
4	19.775	37.85	$C_{18}H_{34}O_2$	282	Oleic Acid	
5	19.925	5.62	$C_{18}H_{36}O_2$	284	Octadecanoic acid	

GC-MS analysis of fraction 19 of *F. racemosa*

The GC-MS chromatogram of anticoccidial active fraction 19 isolated from methanolic leaves extract of *F. racemosa* revealed 5 peaks at different retention times and confirmed the presence of five compounds (Table 5.14). The major compounds were acetone (61.38%), Oleic Acid (24.78%), l-(+)-Ascorbic acid 2,6-dihexadecanoate (8.18%). While Octadecanoic acid (3.63%) and Cyclododecanemethanol (2.02%) are present in minor quantity. The GC-MS chromatogram of fraction 19 is represented in figure 5.14.

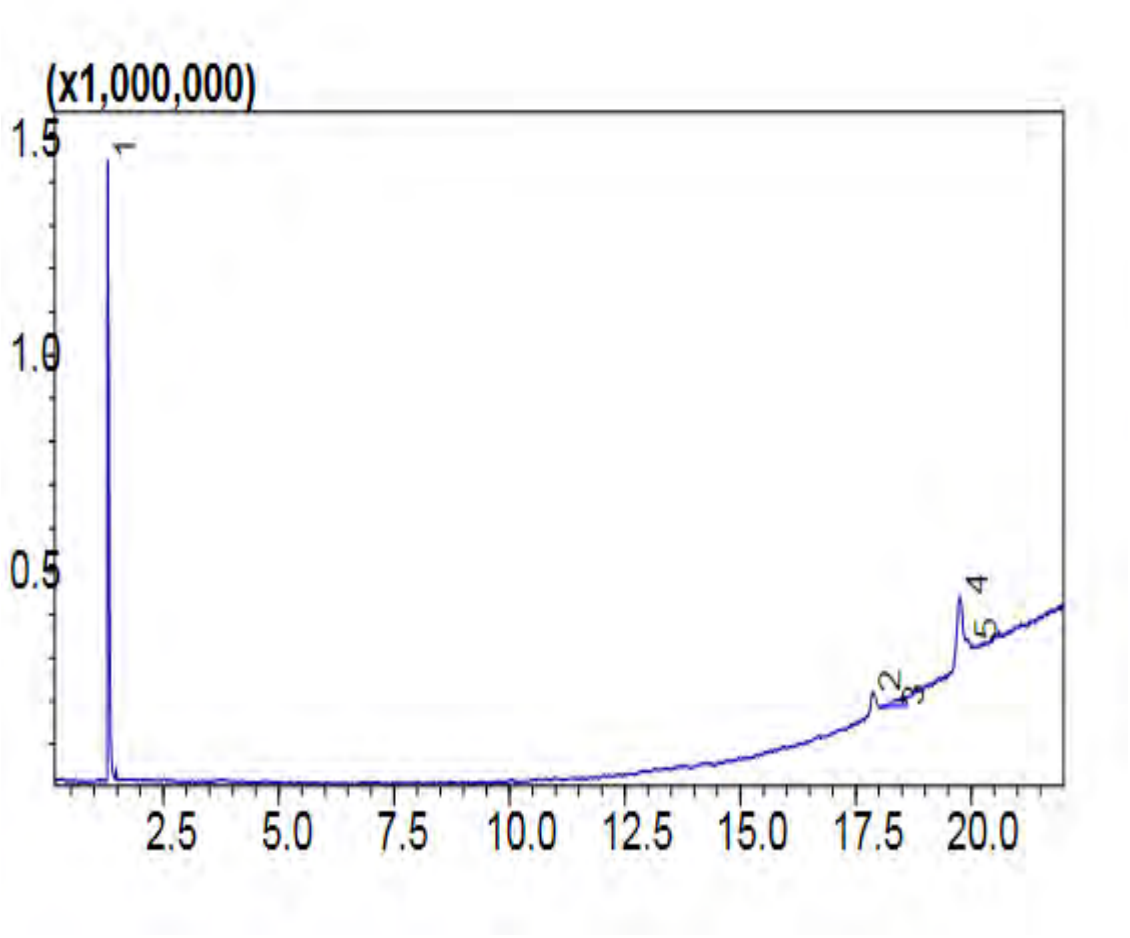
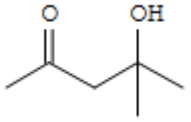
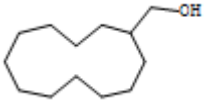
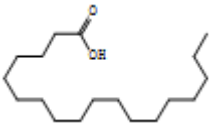


Figure: 5.14: GC-MS chromatogram of fraction 19 of *F. racemose.*

Table 5.14. Spectral data of GC-MS chromatogram of fraction 19 of *F.racemosa*.

S.N	Peak Retention time	Peak Area (%)	Molecular Formula	Molecular weight	Compound analyzed	Compound structure
1	1.296	61.38	C_3H_6O	58	Acetone	
2	17.859	8.18	$C_{38}H_{68}O_8$	652	l-(+)-Ascorbic acid 2,6-dihexadecanoate	
3	18.075	2.02	$C_{13}H_{26}O$	198	Cyclododecane methanol	
4	19.753	24.78	$C_{18}H_{34}O_2$	282	Oleic Acid	
5	19.945	3.63	$C_{18}H_{36}O_2$	284	Octadecanoic acid	

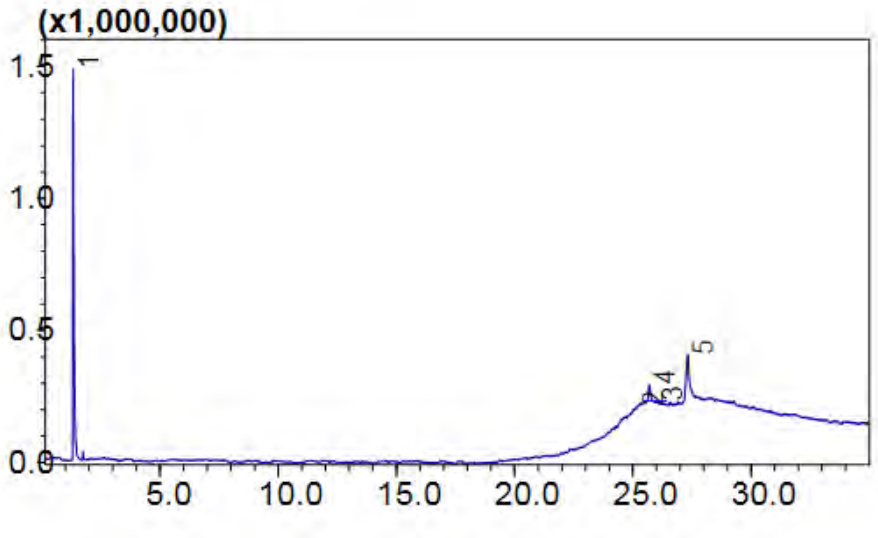
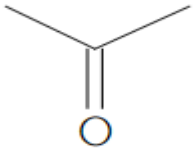
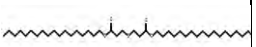
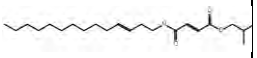
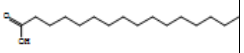
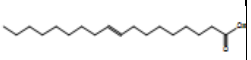


Figure: 5.15: GC-MS chromatogram of fraction 20 of *C. fistula.*

GC-MS analysis of fraction 20 of *C. fistula*

In anticoccidial active fraction 20 of C. fistula leaves extract, peak values of 3333.61, 2946.23 and 2834.89 cm-1 confirmed the presence of N-H / O-H stretching of strong amine salt. Peak values 1650.61 and 1449.55 cm-1 indicate the presence of C-H weak and medium bending of alkane respectively. Peak values of 1409.73 cm-1 confirmed the presence of S=O stretching of sulfonyl chloride. The peak values 1261.09 and 1113.05 cm-1 clearly indicate the presence of C-O strong stretching of aliphatic compounds. The peak value 1017.72 cm-1 confirmed the presence of C-F stretching of fluro compounds. The peak values 580.05 and 462.06 cm-1 indicate the presence of C-Cl and C-H stretching and bending of halo compounds and 1,2,4-trisubstituted respectively (Figure 5.15 and Table 5.15).

Table 5.15. Spectral data of GC-MS chromatogram of fraction 20 of *C.fistula.*

S.N	Peak Retention time	Peak Area (%)	Molecular formula	Molecular weight	Compound analyzed	Compound structure
1	1.325	61.18	C_3H_6O	58	Methyl ketone	
2	25.20	3.69	$C_{42}H_{82}O_4S$	682	Distearyl thiodipropionate	
3	25.510	4.56	$C_{22}H_{38}O_4$	366	Fumaric acid, isobutyl tetradec-3-enyl ester	
4	25.698	11.50	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
5	27.326	19.06	$C_{18}H_{34}O_2$	282	9-Octadecenoic acid	

GC-MS analysis of fraction 22 of *C. fistula*

The GC-MS chromatogram of fraction 22 consist of 5 peaks having Acetone (59.28%) and Oleic Acid (20.21%) as major components while Urs-12-ene (7.56%), l-(+)-Ascorbic acid 2,6-dihexadecanoa (6.95%) and Stigmast-5-en-3-ol, oleate (5.99%) as the minor components (Figure: 5.16 and Table: 5.16).

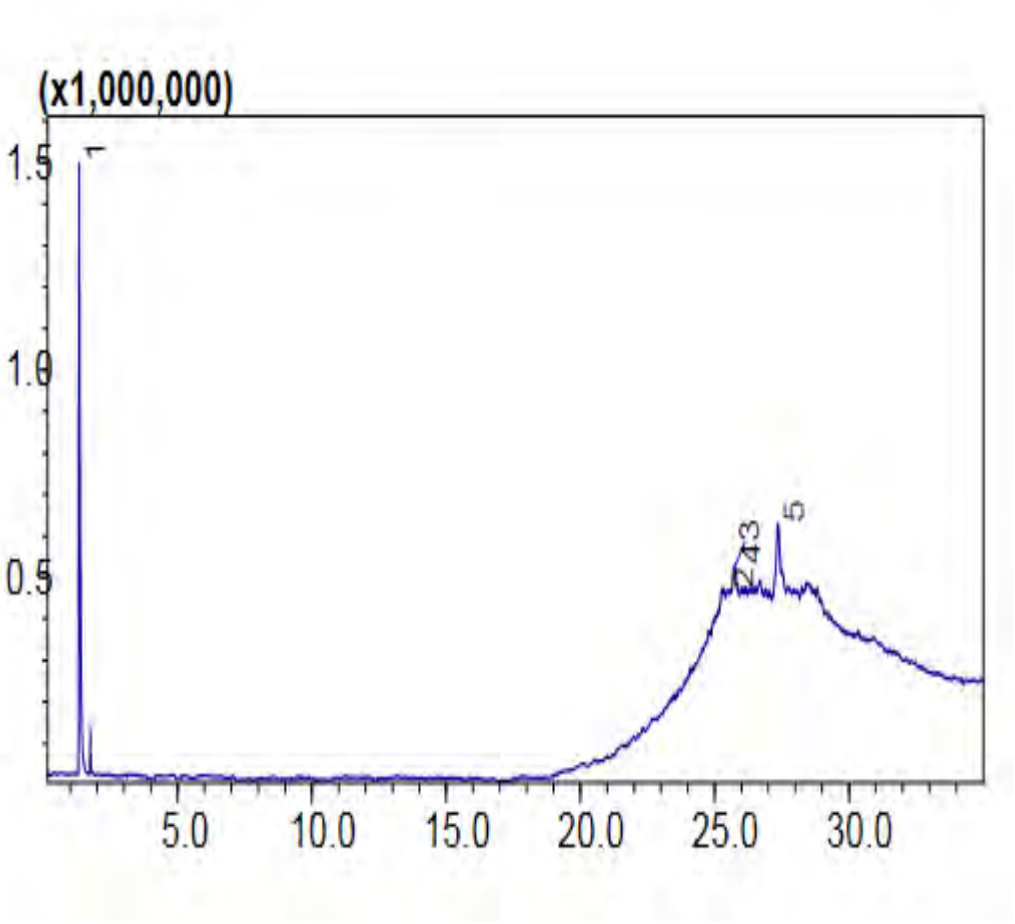
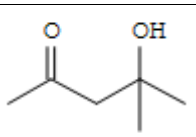
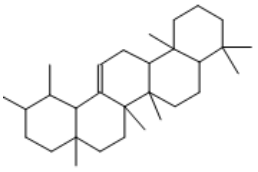
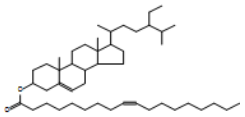
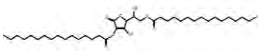
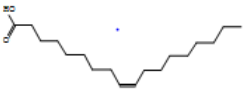


Figure: 5.16: GC-MS chromatogram of fraction 22 of *C. fistula.*

Table 5.16: Spectral data of GC-MS chromatogram of fraction 22 of *C.fistula*

S.N	Peak Retention time	Peak Area (%)	Molecular formula	Molecular weight	Compound analyzed	Compound structure
1	1.33	59.28	C_3H_6O	58	Acetone	
2	25.47	7.56	$C_{30}H_{50}$	410	Urs-12-ene	
3	25.70	5.99	$C_{47}H_{82}O_2$	678	Stigmast-5-en-3-ol, oleate	
4	25.73	6.95	$C_{38}H_{68}O_8$	652	l-(+)-Ascorbic acid 2,6-dihexadecanoa	
5	25.35	20.21	$C_{18}H_{34}O_2$	282	Oleic Acid	

GC-MS analysis of fraction 16 of *S. cumini*

The GC-MS chromatogram of anticoccidial active fraction 16 of *S. cumini* from methanolic leaves extract revealed ten peaks at different retention times and confirm the presence of ten compounds (Table 5.17). Trichloromethane and acetone was the most abundant components having an area percentage of 25.56 and 20.64% respectively. 2-Octanone (19.46%), n-Hexane (17.03), 9-Octadecenoic acid, (E)- (1.33%), Triacontane (2.95), Decanedioic acid, diethyl ester (3.86), n-Hexadecanoic

acid (5.67), Dotriacontane (1.75), Hexadecane, 2-methyl-(1.73) are the minor components. The GC-MS chromatogram of fraction 16 is represented in figure 5.17.

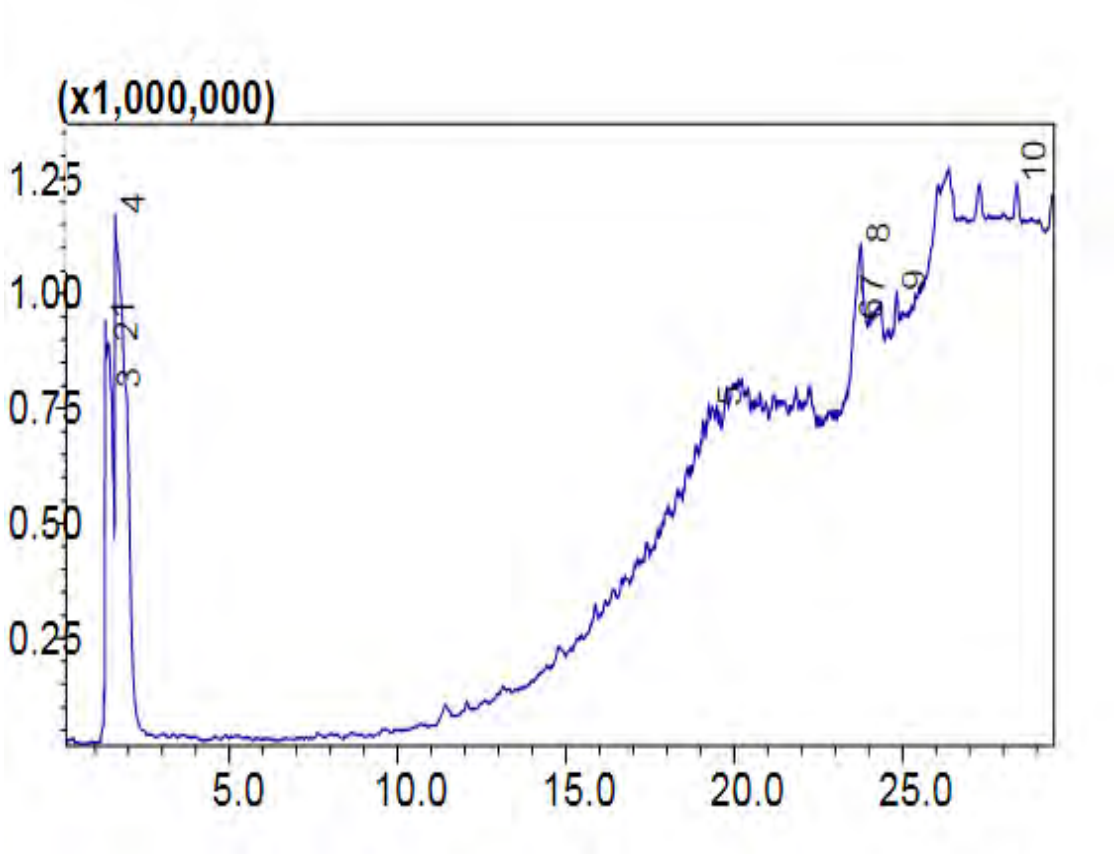
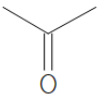
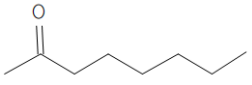
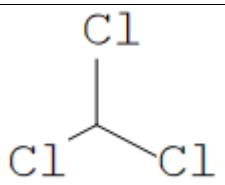
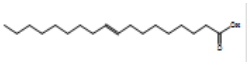
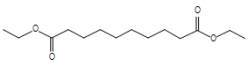
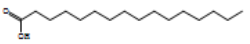
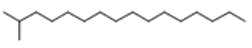


Figure: 5.17: GC-MS chromatogram of fraction 16 of *S. cumini.*

Table 5.17. Spectral data of GC-MS chromatogram of fraction 16 of *S.cumini*

S. No	Peak Retention time	Peak Area (%)	Molecular formula	Molecular weight	Compound analyzed	Compound structure
1	1.313	20.64	C_3H_6O	58	Acetone	
2	1.406	19.46	$C_8H_{16}O$	128	2-Octanone	
3	1.500	17.03	C_6H_{14}	86	n-Hexane	

4	1.608	25.56	$CHCl_3$	118	Trichloromethane	
5	19.359	1.33	$C_{18}H_{34}O_2$	282	9-Octadecenoic acid, (E)-	
6	23.555	2.95	$C_{30}H_{62}$	422	Triacontane	
7	23.615	3.86	$C_{14}H_{26}O_4$	258	Decanedioic acid, diethyl ester	
8	23.761	5.67	$C16H_{32}O_2$	256	n-Hexadecanoic acid	
9	24.834	1.75	$C_{32}H_{66}$	450	Dotriacontane	
10	28.396	1.73	$C_{17}H_{36}$	240	Hexadecane, 2-methyl-	

GC-MS analysis of fraction 19 of *S. cumini*

The GC-MS chromatography of anticoccidial active fraction 19 isolated from methanolic leaves extract of *S. cumini* showed 15 peaks which confirmed the presence of 15 bioactive compounds. The major bioactive compound in fraction 19 are Trichloromethane with peak area percentage of (55.51%). The rest of compounds like Phthalic acid, butyl undecyl ester (3.86%), n-Hexadecanoic acid (2.59%), Phthalic acid, butyl tridecyl ester (2.53%), Phthalic acid, 4,4-dimethylpent-2-yl butyl ester (1.48%) etc are found in less proportion (Figure 5.18: Table 5.18).

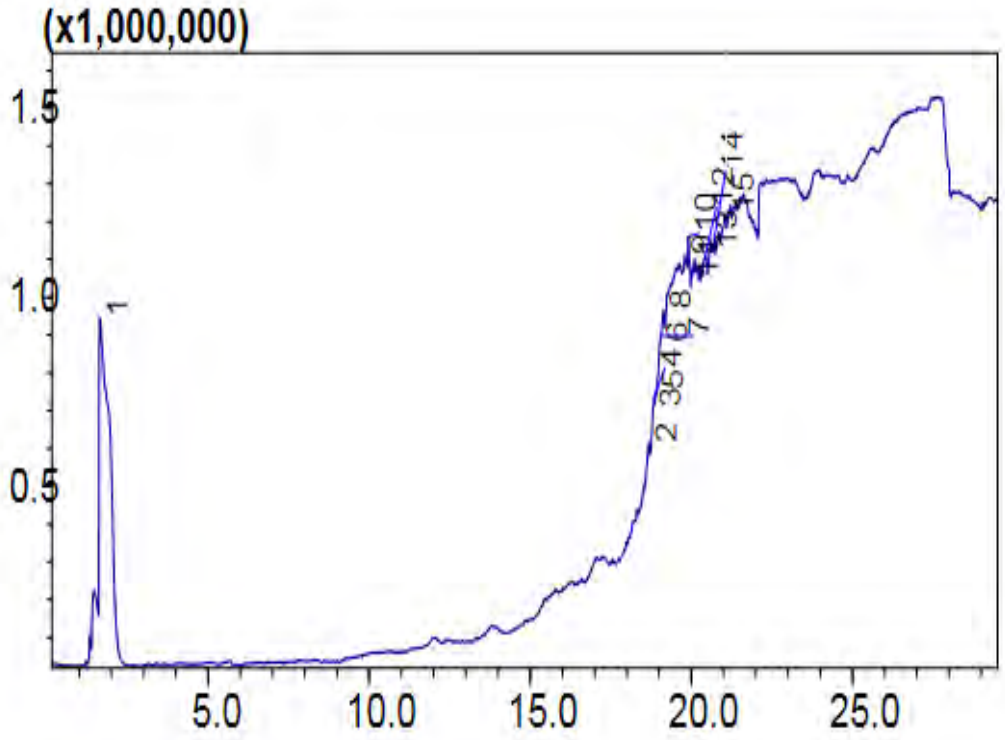
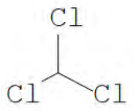
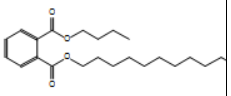
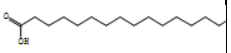
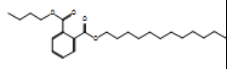
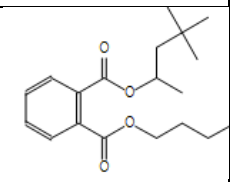
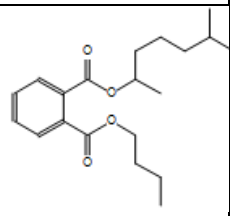
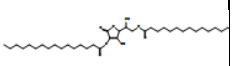
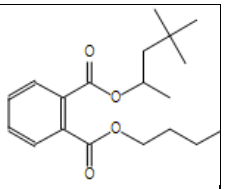
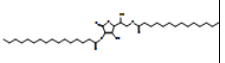
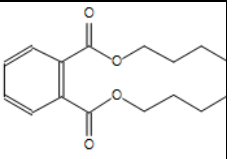
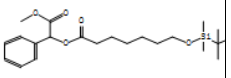
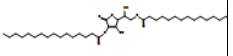
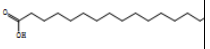
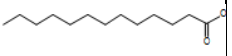
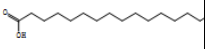


Figure: 5.18; GC-MS chromatogram of fraction 19 of *S. cumini*

Table 5.18. Spectral data of GC-MS chromatogram of fraction 19 of *S.cumini*

S. N	Peak Retention time	Peak Area (%)	Molecular formula	Molecular Weight	Compound analyzed	Compound structure
1	1.640	55.51	$CHCl_3$	118	Trichloromethane	
2	18.680	3.86	$C_{23}H_{36}O_4$	376	Phthalic acid, butyl undecyl ester	
3	18.810	2.59	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
4	18.840	2.53	$C_{25}H_{40}O_4$	404	Phthalic acid, butyl tridecyl ester	
5	18.895	1.48	$C_{19}H_{28}O_4$	320	Phthalic acid, 4,4-dimethylpent-2-yl butyl ester	
6	19.020	3.28	$C_{20}H_{30}O_4$	334	Phthalic acid, butyl 6-methylhept-2-yl ester	
7	19.045	4.25	$C_{38}H_{68}O_8$	652	l-(+)-Ascorbic acid 2,6-dihexadecanoat	

8	19.130	3.98	$C_{19}H_{28}O_4$	320	Phthalic acid, 4,4-dimethylpent-2-yl butyl ester	
9	19.798	1.46	$C_{38}H_{68}O_8$	652	l-(+)-Ascorbic acid 2,6-dihexadecanoat	
10	19.906	6.87	$C_{16}H_{22}O_4$	278	Dibutyl phthalate	
11	19.995	1.45	$C_{22}H_{36}O_5Si$	408	Benzeneacetic acid, .alpha.-(7-t-butyld	
12	20.431	4.80	$C_{38}H_{68}O_8$	652	l-(+)-Ascorbic acid 2,6-dihexadecanoat	
13	20.565	2.36	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
14	20.716	4.04	$C_{13}H_{26}O_2$	214	Tridecanoic acid	
15	21.076	1.54	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	

In Silico Molecular Docking

All of the identified compounds were used as ligands in docking and evaluated on the basis of binding affinities to justify their anticoccidial potential. Among docked compounds, the best E value of compound Oleic acid of F-15 is -5.8 and Cyclododecane methanol of F-19 is -6.5 *Kcal/mol* of *F.racemosa* methanolic crude extract. In current

docking different forces present between the best-docked compounds i.e., oleic acid and Cyclododecane methanol, and the target protein. The two hydrogen bonds present between each compound and target protein. Besides these, some other forces like Vander wall forces, alkyl, and Pi-alkyl interactions are present in the ligand-protein interacted complex. Vander wall forces stabilize the formed complex by the creation of a strong cohesive environment (Figure: 5.19).

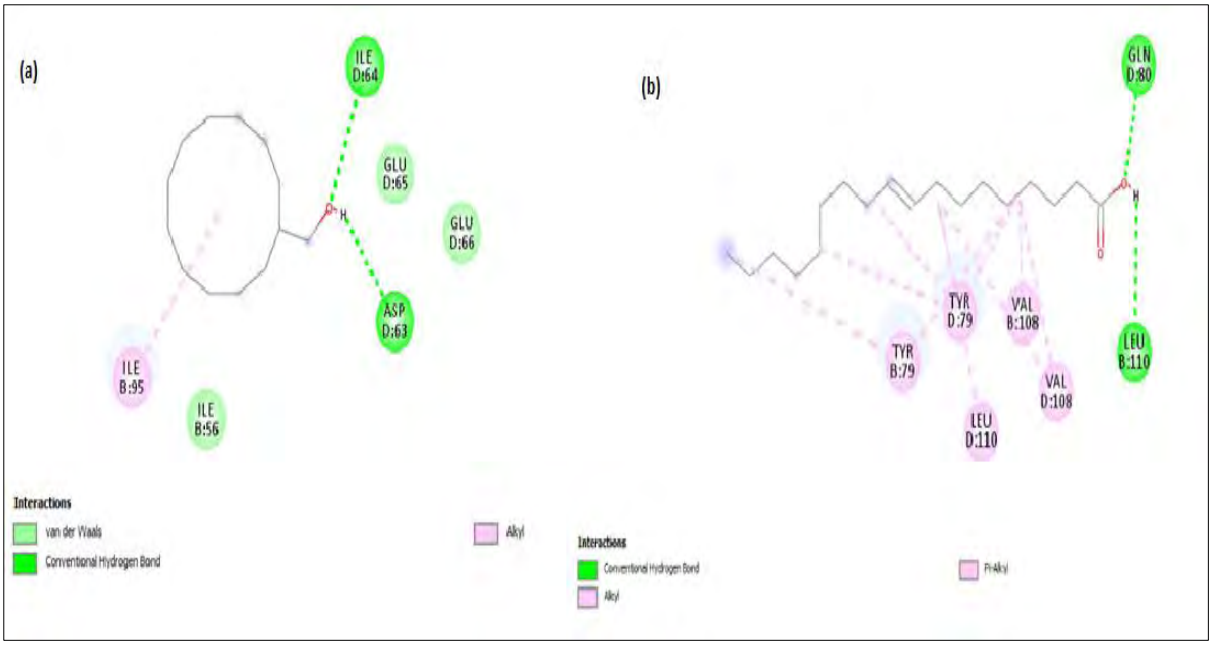


Figure 5.19: Interaction plot of compounds. (a) Cyclododecane methanol (Binding affinity: 6.5 *Kcal/mol*) and (b) oleic acid (Binding affinity:5.8 *Kcal/mol*) with target.

DISCUSSION

The isolation of natural bioactive components from plant extracts aids in the creation of novel medications for the treatment of diseases. Plants are rich source of many phytochemicals that are used in different industries as part of many herbal medicine from crude plant extract. Many plants particularly *F. racemosa*, *C. fistula* and *S. cumini* containing antioxidant, antiparasitic and anticoccidial components. Therefore, in present study, from selected plants like *F. racemosa, C. fistula* and *S. cumini* anticoccidial active fractions were isolated and characterized by UV-Vis spectroscopy, FTIR and GC-MS analysis.

Bioactive fractions UV-Vis spectroscopic analysis of selected plants were performed at 200 to 800nm. In all fractions a common absorption peaks were observed in range of 320-380nm having different absorption spectra. Ray *et al*., (2012) reported similar absorption spectra from *Aloe barbadensis* Miller gel. Furthermore, the absorption band between 320 and 380 nm is thought to be a marker for phenolic chemicals (Ozsoy *et al*. 2008).

Fraction 15 of *F. racemosa* and F-22 of *C. fistula* showed absorption spectra of 660 and 665nm respectively. Jain *et al*., (2016) reported similar absorption spectra at 665nm in *Mentha spicata* plant extract. Rani *et al*., (2016) observed nearest absorption peaks in leaf extracts of *Meizotropis pellita* at 660nm and by Rajeshkumar and Jayaprakash., (2016) at 669.12nm on red seaweed *(Acanthophora specifera).*

The absorption maxima in the UV-Vis spectra of the various anticoccidial fractions of the experimental plant extracts were in the range of 207-390 nm. Flavonoid and its derivatives exhibit these absorption bands as a characteristic. The two absorption peaks in the 230–285 nm and 300–350 nm wavelength ranges define the flavonoid spectrum (Dhivya and Kalaichelvi, 2017). Similar findings were corroborated by a prior work by Rani *et al*., (2016), which showed the UV-Vis spectra of leaf extract in the absorption region between 270 and 340 nm.

Based on the peak values in the infrared radiation band, the FTIR spectrum was utilized to identify the functional groups in the extract. According to the results of the FTIR analysis, there were alcohols, phenols, alkanes, aldehydes, ketones, aromatics,

aliphatic amines, aromatic amines, amides, carboxylic acids, esters, nitro compounds, alkynes, primary and secondary amines, and alkyl halides, alkyl aryl ether, sulfonyl chloride, fluoro and halo compound. Almost similar functional groups are also reported by Paul and Devi., (2021) in *Ficus racemosa* and *Ficus auriculata* methanolic fruit extracts. The major compounds identified in different anticoccidial active fractions of *F. racemosa* are Cyclododecanemethanol

On GC-MS analysis of *F. racemosa* anticoccidial F-15, the confirmed bioactive compounds were acetone (48.47%), Oleic Acid (37.85%), n-Hexadecanoic acid (7.60%), Octadecanoic acid (5.62%), Trichloromethane (0.46%) etc. while in F-19 the confirmed compounds were Oleic Acid (24.78%), l-(+)-Ascorbic acid 2,6-dihexadecanoate (8.18%), Octadecanoic acid (3.63%), Cyclododecanemethanol (2.02%) and etc. N-hexadecanoic acid (also known as palmitic acid) is one of the compounds found in the *F. racemosa* fruit methanol extract, was also reported to be present (Paul and Devi., 2021). n-Hexadecanoic acid have anti-inflammatory, antioxidant, hypocholesterolemic, anti-androgenic, and hemolytic properties (Aparna *et al*., 2012; Irulandi *et al*., 2016).

l-(+)-Ascorbic acid 2,6-dihexadecanoate identified in anticoccidial fractions of *F.racemosa* leaves extract, is a vitamin well-known for its anti-inflammatory, anti-cancer, and anti-infertility activities (Kadam and Lele, 2017; Ramya *et al*., 2015). In previous studies Ascorbic acid were identified in *F. racemosa*, *F. auriculata* and *Bryonopsis laciniosa* fruits methanol extracts (Ramya *et al*., 2015; Paul and Devi., 2021). The biological properties of linoleic acid or 9, 12- octadecadienoyl chloride, (z,z), include anti-inflammatory, anti-cancer, anti-eczemic, anti-coronary, insectifugal, and nematicidal (Olaleye *et al*., 2018).

Almost similar phytochemicals were identified through GC-MS analysis of *F. racemosa* bark extract (Rathinavel *et al*., 2019). For docking the enzyme S-adenosylmethionine synthase that play important role in life cycle of coccidiosis causing agent *Eimeria* was selected as a target. In binding complexes different bonding forces like hydrogen, alkyl and Pi-alkyl, Vander wall forces and etc play crucial role. Due to their role in facilitating protein-ligand interaction and being crucial for protein folding, hydrogen bonds play an important role in docking (Salentin *et al*., 2014). Alkyl

and Pi-alkyl interactions are also crucial for charge transfer, which aids in inserting the drug into the receptor's binding site (Arthur and Uzairu., 2019).

Conclusion

These phytocompounds identified through different phytochemicals techniques in selected plants anticoccidial fractions reported to have wide range of bioactive properties such as anti parasitic, anti microbial, anti oxidant, anti inflammatory etc. The presence of such bioactive compounds in the studied plants justifies their use in the traditional medicine system. The compounds Oleic acid and Cyclododecane have highest binding affinity on in silico molecular docking. Therefore, highly recommended to exploit its immense potential and use as lead compounds against coccidiosis at the commercial level in broilers.

General Discussion

GENERAL DISCUSSION

The protozoan parasites of the genus *Eimeria* that live and reproduce in the gut cause coccidiosis. These parasites led to an intestinal illness in chicks which led to financial losses for the global poultry sector (El-Shall *et al*., 2022). The disease is significant economically because of the production losses, severe morbidity, and high fatality rates brought on by acute, bloody enteritis. The intestinal lesions of infection vary amongst *Eimeria* species because they have different sites of infection in the digestive system (Blake *et al*., 2020). Coccidiosis is considering as the most damaging disease, with an annual loss of US$ 127 million (El-Shall *et al*., 2022). In only seven countries, about £7.7 to £13.0 billion is spent by the poultry industry on avian coccidiosis prevention., treatment, and production losses each year. Seven *Eimeria* spp are responsible for these losses. Within the digestive tract of broilers, the species grows in different places (Blake *et al*., 2020). The intestinal mucosa of several mammals and birds is affected by almost 1800 different *Eimeria* species, however the seven *Eimeria* species that cause coccidiosis in chicks are *E. tenella*, *E. necatrix*, *E. acervulina*, *E. maxima*, *E. brunetti*, *E. mitis*, and *E. praecox*. The direct life cycle, fecal-oral transmission, presence of resistant oocysts, lack of cross-protection between *Eimeria* species, high oocyst reproductive potential, high stocking density, and favourable environmental conditions for infectivity are just a few of the factors that aid in the development of coccidiosis. Nearly three-quarters of the overall economic expenses are due to subclinical coccidiosis, which is the most serious issue. Due to higher feed consumption and slower body weight gain, flock performance is poor (Remmal *et al*., 2011).

Due to the development of significant anticoccidial resistance in *Eimeria* species and the issues associated with medication residues, this topic is particularly crucial (Hashemi and Davoodi, 2010). In the poultry industry around the world, a number of chemotherapeutic agents are used. On the wellbeing and productivity of broilers, several risky medication effects have also been documented. Anticoccidial medications such as polyether ionophores, sulfanamides, and chemical medicines are prophylactically administered via feed to prevent coccidiosis and reduce associated economic losses (De Gussem, 2007). Due to their regular use, *Eimeria* strains have become drug-resistant (Peek and Landman, 2003). Moreover, there is strong evidence

that residues of some coccidiostats may be present in the meat and egg and the consumer is not adequately protected (Olejnik *et al*., 2009). People are considering alternatives because of the risks that come with chemotherapeutic drugs and antimicrobial resistance. Therefore, alternative strategies for coccidiosis control are needed besides the application of coccidiosis vaccines that are expensive. In this regard, herbal compounds seem to have some potential in controlling coccidiosis (Abbas *et al*., 2012). Herbal remedies have been used in medicine for as long as human history. They have recently gained increasing popularity especially because of the declining effectiveness of synthetic compounds in addition to concerns of consumers about drug effects (Kim *et al*., 2013).

In current study aqueous and methanolic leaves extract of three plants i.e., *Ficus racemosa*, *Cassia fistula*, and *Syzygium cumini* were investigated for their anticoccidial and antioxidant potential against mixed *Eimeria* species. Oocysts sporulation inhibition and sporozoite viability inhibition assays were carried out for the evaluation of *in vitro* anticoccidial efficacy of extracts. The set up was examined after 48 hours of incubation. For the evaluation of the antioxidant potential of extracts, DPPH radical scavenging activity, ferric reducing antioxidant power and total antioxidant capacity were carried out. Preliminary phytochemical screening of selected plants extract was performed for the detection of various antioxidant compounds. *C. fistula* leaves methanolic extract showed maximum oocysts sporulation inhibition (86.81±2.35%) and sporozoites viability inhibition (86.73±1.67%) at concentration 30*mg/ml* against *E. mitis* and *E. tenella* respectively. Similar results were observed by Desalegn and Ahmed, (2020). They reported that the aloe species anticoccidial effect was increased with increasing concentrations.

The highest radical scavenging capacity (67.82±0.00) and reducing power (2.17±0.01) were shown by *F. racemosa* and *C. fistula* methanolic leaf extract respectively. Maximum total antioxidant power was observed in *C. fistula* (30.95±0.35) *µg/mg* methanolic leaf extracts. *C. fistula* methanolic extracts having maximum amount of phenols (32.50±0.00) and flavonoids (40.00±1.00)*µg/ml*. Similar to our findings Deeksha and Arunachalam, (2019) also reported maximum TP and TF contents in *C. fistula* extract. Various bioactive compounds like phenols, flavonoids, alkaloids, saponins, terpenoids and etc commonly used as medicinal attributes were confirmed in

all selected plants. Bagyalakshmi *et al*., (2019) confirmed tannins, alkaloids, flavonoids, saponins etc on qualitative phytochemical screening of *F. racemosa* extract. Pavai *et al*., (2019) in *C. fistula* and Ramos and Bandiola, (2019) in *S. cumini* reported different bioactive chemicals. Which is consistent with the current study. The current study's findings showed that experimental plants methanolic extracts had the highest anticoccidial and antioxidant effects because they contain medicinally significant phytochemicals. Therefore, further studies are carried out for characterization, fractionation and identification of anticoccidial fractions from selected plants methanolic leaves extracts by column and thin layer chromatography. Column chromatography has certain advantages over other analytical techniques being less expensive, avoid cross contamination and easy disposal and degradation of stationary phase (Abdulhamid *et al*., 2017). In the present study crude methanolic leaves extract of each selected plant were fractionated by column chromatography using step wise gradient technique in different solvents. Similarly, Shabrina *et al*., (2018) followed the same pattern of increasing polarity in mobile phase isolating *Garcinia fruticosa lauterb* extract fractions during column chromatography. Collected fractions were further finalized by thin layer chromatography (TLC). Total of 18 fractions from *F. racemosa*, 23 fractions from *C. fistula* and 19 fractions from *S. cumini* were eluted with different *Rf* values. Oocysts sporulation inhibition and sporozoites viability inhibition assays were carried out for the evaluation of *in vitro* anticoccidial activities of eluted fractions. Each eluted fraction qualitative, quantitative phytochemical screening and antioxidant potential were also examined.

Among all the eluted fractions, maximum sporulation inhibition (76.56±1.14%) was shown by F-19 at a concentration of 30*mg/ml*. Similarly, Abbas *et al.,* (2019) revealed that *T. ammi* extract exhibited *in vitro* anticoccidial activity by influencing *Eimeria* oocyst sporulation in a dose-dependent manner. Similarly, Cedric *et al*., (2018) reported that *P. guajava* extracts inhibit growth and development of oocysts at maximum concentration of 30*mg/ml*. During antioxidant potential estimation, maximum DPPH radical scavenging inhibition and total antioxidant capacity was shown by F-15 of *F. racemosa*. While the highest ferric reducing power was recorded in F-19 of *S. cumini* at a concentration of 600*µg/ml*. Among all the eluted fractions of

selected plants, maximum total phenolic and flavonoid contents were recorded in F-22 of *C. fistula.*

From the statistical analysis of the present study it is cleared that there is a significant correlation between TPC, TFC and antioxidant activities. Numerous studies have been published that demonstrate the association between TPC, TFC, and other antioxidant activities (Manzoor *et al*., 2012). Various fractions of all selected plants possess the maximum tested phytochemicals. In preliminary phytochemical investigations the methanolic extract of selected plants fractions indicated the presence of tannins, phenols, alkaloids, glycosides, steroids, terpenoids, phenolic compounds, and cardiac glycosides (Gowri and Vasantha., 2010). Crozier *et al*., (2008) reported that the saponins from plants had antibacterial and anti-inflammatory properties. The tannins were shown to have antimicrobial, antimutagenic, anticarcinogenic, antidiarrheal, and antiseptic activities in earlier investigations (Graça *et al*., 2016). Lakshmi *et al*., (2020) proposed that *F. racemosa* extract contains a wide range of phytochemicals. The findings of the current study are consistent with the research of Mongkolvai *et al*., (2021), who suggest that *C. fistula* leaves contain more phytochemicals than fruit extract. Similarly, a study conducted by Khan *et al*., (2017) on the *C. fistula* extract revealed that phyto-constitutional investigation based on chemical testing revealed the presence of flavonoids and saponin, tannin, glycoside, and alkaloid in both methanolic and aqueous extract, respectively.

In present study several fractions of selected plants possess best anticoccidial and antioxidant properties because of the presence of medicinally significant phytochemicals. As a result, more research evaluated the *in vivo* anticoccidial efficacy of active fractions of methanolic extract of experimental plants on chicks infected with mixed species of *Eimeria.* Thus, the anticoccidial activity of different concentrations of crude extracts of *F. racemosa*, *C. fistula*, *S. cumini* and their fractions was tested against mixed *Eimeria* species in broiler chicks (n = 315) in comparison with anticoccidial amprolium. On day 14th, the experimental birds were allotted 21 groups designated as A-U. Each group was in triplicate with 5 chicks per group. The chicks in all experimental groups except negative control group T were orally infected with 7000 to 10,000 sporulated oocysts of mixed *Eimeria* species. On day 4th post inoculation (PI), as the first sign of infection appeared, all groups except negative control group T and

medicated group S were treated with different supplements of plant extracts and their fractions orally at a dose of 1 ml/chick daily for 5 days using a dropper. Medicated group S was given amprolium (1.25mg/ml) in feed. The effectiveness of experimental doses was evaluated for 36 days on the basis of factors such as average weight increase, feed intake, feed conversion ratio, oocyst shedding, diarrhea, mortality, blood biochemistry, haematology, histopathology, and other variables. The acute toxicity of extracts was investigated by using 50one-day old broiler chicks. A total of 10 groups with 5 chicks in each. A maximal oral dosage 2,000 mg kg-1 b.wt of different extracts of selected plants and their anticoccidial fractions was given to all groups except control group were given. All the chicks were observed over 14 days for any clinical signs of toxicity. Drug resistance of *Eimeria* species was assessed by using anticoccidial sensitivity test (AST), percent optimum anticoccidial activity (POAA) and anticoccidial. Among all experimental groups, maximum weight gain was recorded on the 5th week in group N (447±4.04) and M (444±3.05) gm treated with (20%) and (10%) of F-22 of *C. fistula* respectively, which was in a similar range to the weight gain of the group treated with amprolium (443±3.07) gm and the uninfected non-medicated control group (456±2.51) gm. Similarly, a study carried out by Ibrahim *et al*., (2016) on anticoccidial effect of different fractions of *Solanum dasyphyllum schumach* and *thonn* crude methanolic extract observed that of the six fractions of *S. dasyphyllum*, fractions 6 and 9 were more effective than amprolium at inhibiting sporulated oocysts of coccidia.

Maximum mean feed consumption was recorded at 5th week of age in groups N (761±0.57) gm and M (759±0.57) gm treated with (20%) and (10%) of F-22 of *C. fistula* respectively, which was higher than the mean feed consumption of groups treated with amprolium (725±1.52) gm and in the range of uninfected non-medicated control group S(762±1.15) gm. Similarly, a study conducted by Christaki *et al*., (2004) proposed that The negative impact of *E. tenella* infection on broiler performance was highlighted by a substantial reduction in body weight growth and feed intake as well as an increase in feed conversion ratio values. However, there were no significant differences between the Apacox levels for feed intake or body weight gain. The best FCR results were shown by group N. The results of this investigation are similar to the work of Habibi *et al*., (2014) that the best treated groups showed lower FCR than other experimental groups.

Among all experimental groups, the greatest decrease in oocyst shedding was recorded in group M treated with 10% of F-22 of *C. fistula*. Abbas *et al.,* (2006) reported reduction in oocysts per gram using ethanolic and aqueous extracts of *Azadirachta indica* fruits and their powdered form. The best-treated groups had no mortality and only mild bloody diarrhea were observed. Similar results of mild bloody diarrhea were shown by studies of Youn *et al.,* (2001) and Kostadinovic *et al.,* (2012). The intestine was severely inflated even during post-mortem, and haemorrhages could be noticed when visually observing the gut without opening it. In the early stages of infection, the intestine was consistently detected to be edematous, thickened, displaying intestinal epithelial necrosis and sloughing, and friable (Sharma *et al*., 2015).

Normal histology, serum biochemistry, and hematology were observed in groups treated with different concentrations of anticoccidial fractions of selected plants. Badria *et al*., (2017) also reported that herbal treated groups in his experiment showed normal histology of the caecum. Mikail *et al*., (2019) also observed the same histopathological changes in liver of coccidia infected chicks as like our study. A significant decrease in Albumin and ALP level of *Eimeria* infected groups was observed which was similar to the study of Mondal *et al*., (2011) and Patra *et al.,* (2010) respectively.

While the level of AST and ALT (Hirani *et al*., 2007), creatinine (Youssef *et al*., 2013) and uric acid (Patra *et al.,* 2010) increased in diseased groups as compare to normal and herbal treated groups. The values of certain haematological markers, such as MCV, MCH, and MCHc, were below the normal limits in chicks infected with *Eimeria* species, which is consistent with other reports (Weiss and Wardrop, 2010; Akhtar *et al*., 2015).

Till the 14th day of the acute toxicity study, there was no mortality. While chicks administered with *F. racemosa* and *C. fistula* crude extracts showed tremors, lethargy, etc. Similarly acute oral toxicity study of *C.fistula* methanolic extract with dose of 5000mg/kg on mice was conducted by Subramanion *et al*., (2011) resulted in no mortalities and behavioral changes in experimental animals. Groups A and S in the current investigation showed substantial drug resistance, according to an assessment of overall drug sensitivity based on a combined analysis of the three indices, B and Q showed moderate resistance, groups E-J, P showed slight resistance, and the rest of the

groups showed sensitivity to their respective treatments. Similarly, a study conducted in China where severe resistance to Amprolium hydrochloride, Toltrazuril and Sulfaquinoxaline sodium was reported by Lan *et al*., (2017).

The anticoccidial active fractions of each selected plant species methanolic extracts were tested for phytochemicals. Characterization by different spectroscopic techniques provides considerable knowledge about the extent and nature of the chemical substances present in extracts. UV-vis spectroscopy, FTIR and GC-MS were used for the characterization of most effective fractions of *F. racemosa.* The identified compounds of each selected fraction were docked with target S-Adenosylmethionine synthase by in silico molecular docking techniques. The UV-vis spectroscopic analysis of anticoccidial fractions was performed at 200 to 800nm and at different wavelengths and different absorption peaks were recorded. Phenolic chemicals are present as indicated by their absorption band between 320 and 380 nm (Ozsoy *et al*. 2008). The FTIR analysis was performed for the identification of different functional groups based on characteristic peak values and the spectrum was recorded by an FTIR spectrometer with a 400 to 4000 cm-1 wavelength scan range. The FTIR analysis of selected fractions of each experimental plant confirmed the presence of amino acid, alkanes, alkynes, amines, aldehydes, carboxylic acid and etc. Almost similar functional groups are also reported by Paul and Devi., (2021) in fruit methanolic extract of *Ficus racemosa* and *Ficus auriculata*. The results of their GC-MS analysis revealed the presence of some common chemical compounds including Oleic Acid, n-Hexadecanoic acid, Octadecanoic acid, Trichloromethane etc (Aparna *et al*., 2012; Irulandi *et al*., 2016). Among all docked compounds cyclododecane methanol of F-19 and oleic acid of F-15 of *F. racemosa* have highest binding affinity with target S-Adenosylmethionine synthase. These compounds are ideal for the synthesis of plants derived anticoccidial medicines. For docking the enzyme S-adenosylmethionine synthase that play important role in life cycle of coccidiosis causing agent *Eimeria* was selected as a target. In binding complexes different bonding forces like hydrogen, alkyl and Pi-alkyl, Vander wall forces and etc play crucial role. Hydrogen bonds have a vital role in protein folding and protein-protein binding, which makes them crucial to docking (Salentin *et al*., 2014). Alkyl and Pi-alkyl interactions are also important for charge transfer, which helps in inserting the drug into the receptor's binding region (Arthur and Uzairu., 2019).

GENERAL CONCLUSION

Anticoccidial medications have often been used as feed additives to control avian coccidiosis. However, it has been noted that widespread usage of anticoccidial medications as a result of which resistant *Eimeria* strains have emerged. Therefore, researchers have focused on alternatives as a means of coccidiosis management to anticoccidial medications. It has been suggested that using botanicals is a possible option in this case. The present study's findings showed that as compared to aqueous extracts, the methanolic extracts of *Ficus racemosa*, *Cassia fistula* and *Syzygium cumini* have strong anticoccidial and antioxidant properties because they contain medicinally important phytochemicals. All the tested plants methanolic extracts showed *in vitro* anticoccidial activities to varying degree but *C. fistula* extract possess maximum oocysts sporulation inhibition and sporozoites viability inhibition at 30*mg/ml* concentration against *E. mitis* and *E. tenella* respectively. *C. fistula* and *F. racemosa* methanolic leaf extract was found to be the best antioxidant due to their maximum reducing power and total antioxidant capacity. In the methanolic extract of all the selected plants, antioxidant substances such phenols, flavonoids, alkaloids, saponins, polysaccharides, etc. were found. The best alternatives to synthetic anticoccidials are these biochemicals.

According to the present findings, all fractions of the experimental plants methanolic extracts showed *in vitro* anticoccidial and antioxidant activities to varying degree with 6 most bioactive fractions, 2 from each plant i.e. F-15 and F-19 of *F. racemosa,* F-20 and F-22 of *C. fistula* and F-16 and F-19 of *S. cumini.* These bioactive fractions possess a variety of phytochemical components. These findings may contribute to the development of a potent substitute for the current anticoccidial medications. Although several anticoccidial drugs are in use, which have side effects and are cost-effective, there is a need for more anticoccidial agents. Hence for further assessment the *in vivo* anticoccidial activity of different concentrations of crude methanolic extracts of *F. racemosa*, *C. fistula*, *S. cumini* and their fractions was tested against mixed *Eimeria* species in broiler chicks (n=315) in comparison with anticoccidial amprolium. On day 14th, the experimental birds were allotted 21 groups designated as A-U. Among all experimental groups, maximum weight gains and mean feed consumption was recorded on the 5th week in group N and M treated with (20%)

and (10%) of F-22 of *C. fistula* respectively, which was in the range of the group treated with amprolium and the uninfected non-medicated control group. All of the fractions treated groups showed best FCR as like control and medicated groups. Group M treated with 10% of F-22 of *C. fistula* showed the greatest decrease in oocyst shedding across all experimental groups. No mortality and diarrhea were observed in the fractions treated groups. Normal histology, serum biochemistry, and hematology were observed in groups treated with different concentrations of anticoccidial fractions of selected plants. In the acute toxicity study, till the 14th day, there was no mortality. Whereas chicks were given *F. racemosa* and *C. fistula* crude extracts showed tremors, lethargy, etc. Groups A and S in the current investigation showed significant drug resistance, according to an estimate of total drug sensitivity based on a combined analysis of the three indices, B and Q showed moderate resistance, groups E-J, P showed slight resistance, and the rest of the groups showed sensitivity to their respective treatments.

For the identification of chemistry of the anticoccidial active fractions, they were subjected to phytochemical characterization. UV-Vis spectroscopy, FTIR and GC-MS were used for the phytochemical characterization of the most effective fraction of *F. racemosa*, *C. fistula* and *S. cumini*. These spectroscopic techniques provide considerable knowledge about the extent and nature of the chemical substances present in these fractions. The identified compounds of each selected fraction were docked with target S-Adenosylmethionine synthase by in silico molecular docking techniques. The UV-vis spectroscopic analysis of anticoccidial fractions was performed at 200 to 800nm and at different wavelengths and different absorption peaks were recorded. The FTIR analysis was performed for the identification of different functional groups based on characteristic peak values and the spectrum was recorded by an FTIR spectrometer with 400 to 4000 cm-1 wave length is the scan range. The FTIR analysis of selected fractions of each experimental plant confirmed the presence of amino acid, alkanes, alkynes, amines, aldehydes, carboxylic acid and etc. According to their GC-MS analysis, the presence of some common chemical compounds including Oleic Acid, n-Hexadecanoic acid, Octadecanoic acid, Trichloromethane etc. Among all docked compounds cyclododecane methanol of F-19 and oleic acid of F-15 of *F. racemosa* have highest binding affinity with target S-Adenosylmethionine synthase. These compounds are ideal for the synthesis of plants derived anticoccidial medicines. These

phytocompounds identified through different phytochemicals techniques in selected plants anticoccidial fractions are said to contain a variety of bioactive qualities, including those that are anti-parasitic, anti-microbial, anti-oxidant, and anti-inflammatory.

Recommendations

The present research work explores the profile of high value phyto-compounds in the anticoccidial active fractions of *F. racemosa*, *C. fistula* and *S.cumini* collected from their natural habitat. A variety of bioactive compounds can be isolated in further research, and *in vivo* models can be used to examine their potential biological principles. Encouraging results recorded in the present study point to importance of plants having their diverse pharmacological value; therefore, inventory of such plants used in ethno veterinary medicine may open further areas of investigations on crude and/or refined drug development.

The compounds Oleic acid and Cyclododecane have highest binding affinity on in silico molecular docking. Therefore, highly recommended to exploit its immense potential and use as lead compounds against coccidiosis at the commercial level in broilers. Detailed studies are needed to isolate, characterize and evaluate these compounds.

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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).

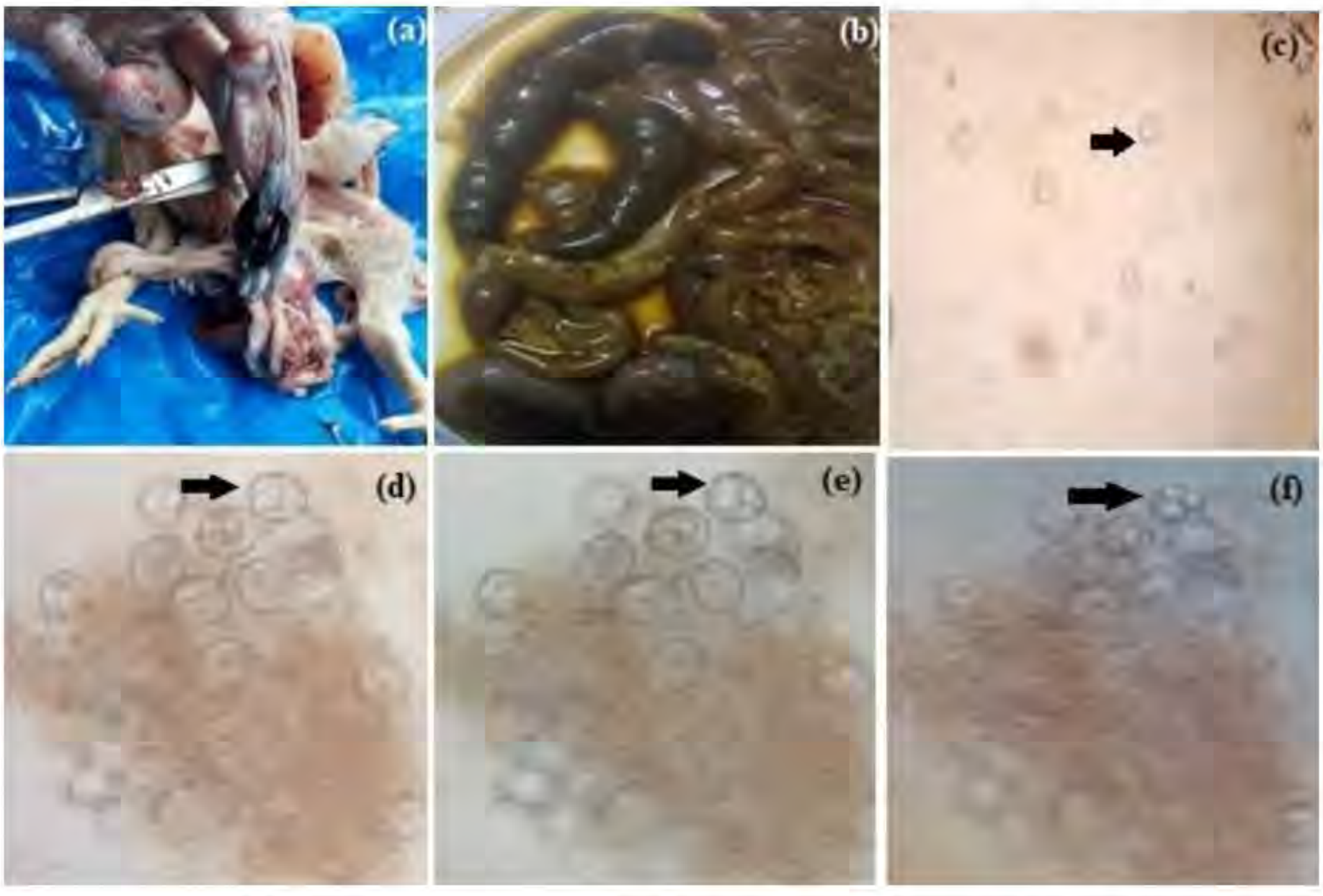

Fig: 1: (a) Coccidiosis infected chick **(b)** Caecum and intestine of infected chick **(c)** un sporulated oocysts **(d), (e)** and **(f)** Sporulated oocysts.

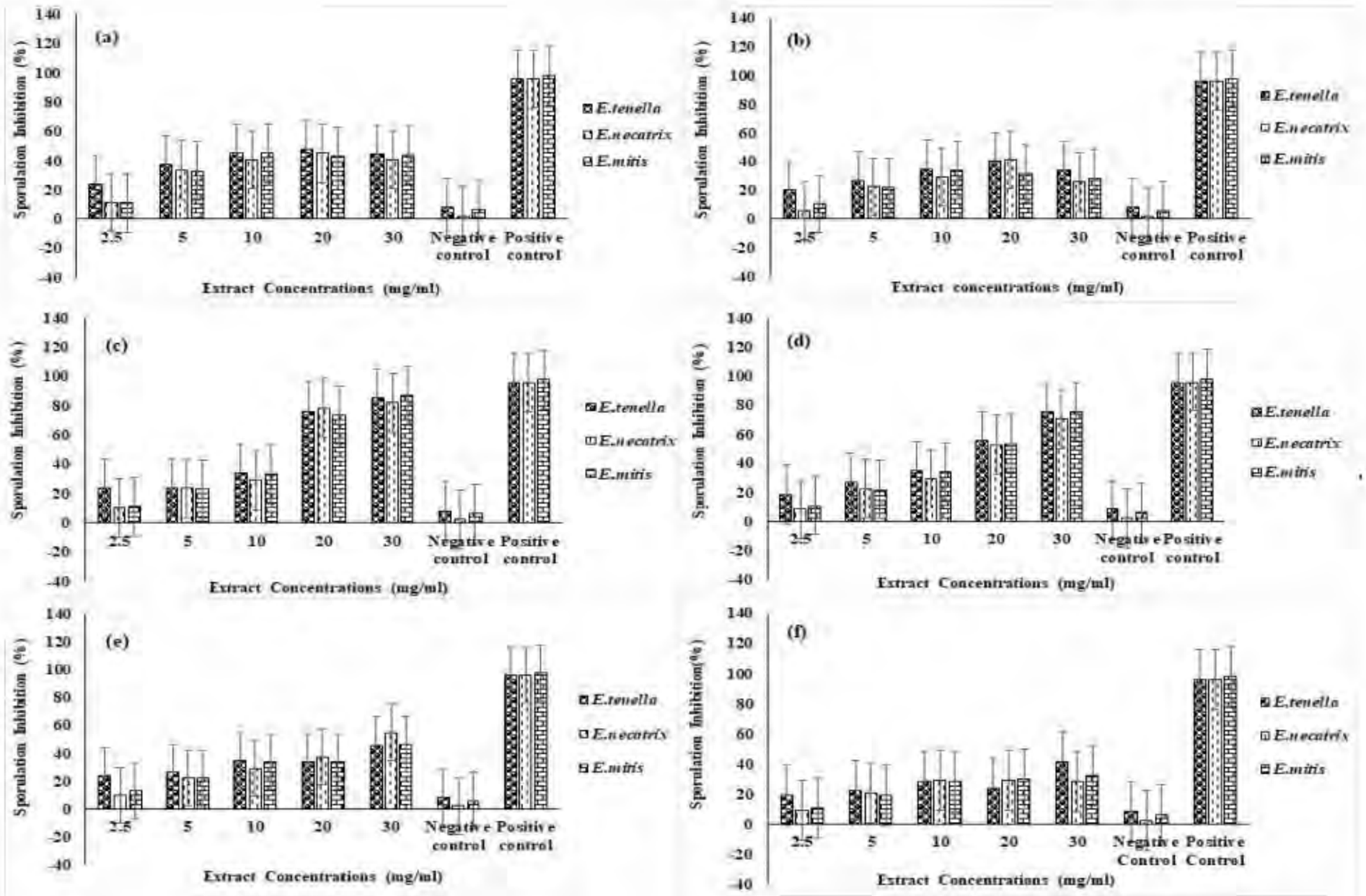

Fig 2: (a), (b): Effect of *F. racemosa* methanolic and aqueous leaves extracts different concentration on sporulation inhibition of different *Eimeria* species oocysts. **(c), (d).** Effect of *C. fistula* methanolic and aqueous leaves extracts different concentration on sporulation inhibition of different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

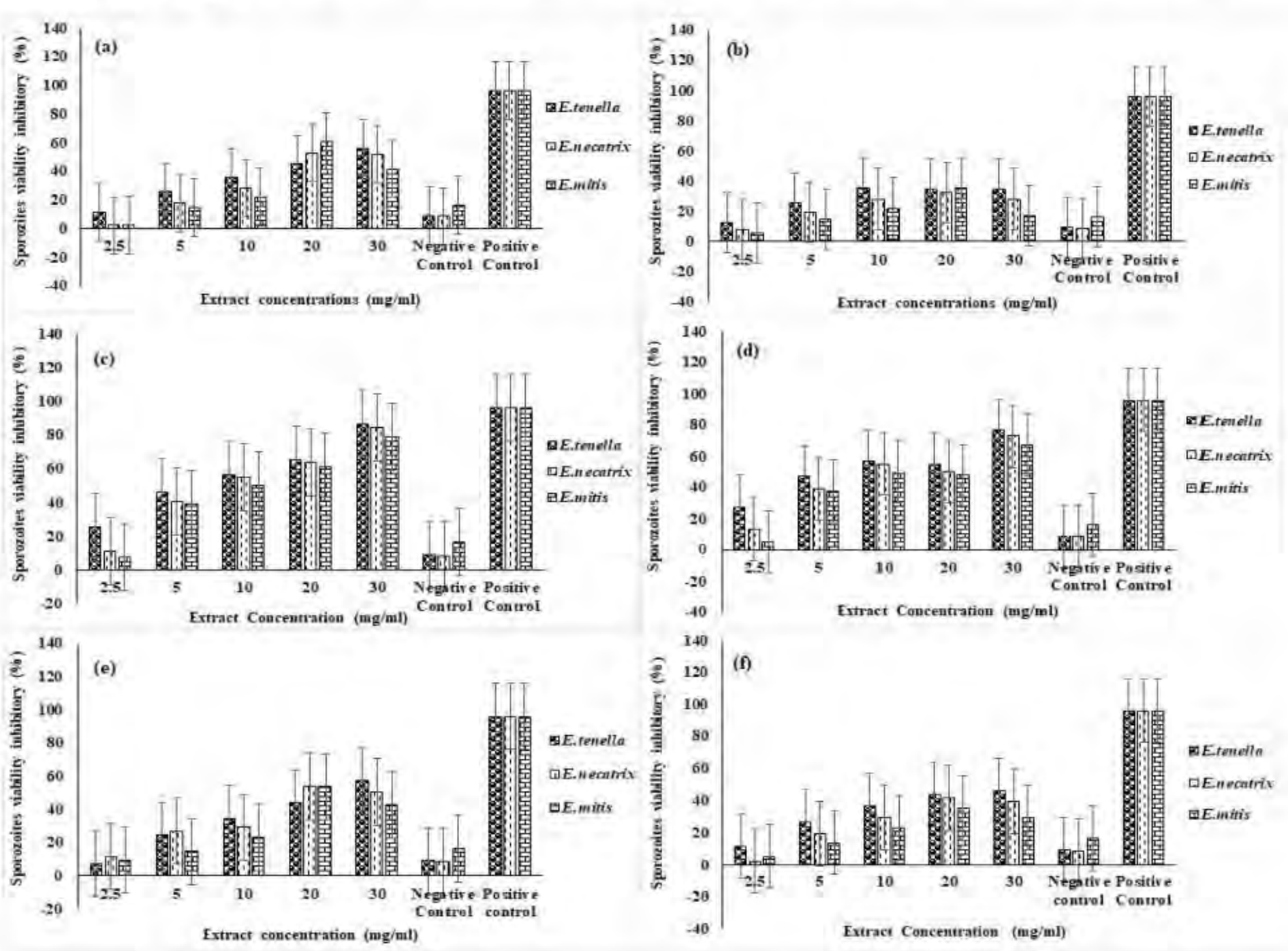

Fig 3: (a), (b): Effect of *F. racemosa* methanolic and aqueous leaves extracts different concentration on sporozoites viability inhibition of different *Eimeria* species. **(c), (d).** Effect of *C. fistula* methanolic and aqueous leaves extracts different concentration on sporozoites viability inhibition 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	Methanol	150	6.31	4.2
racemosa	Water	150	3.99	2.66
Cassia	Methanol	150	5.65	3.76
fistula	Water	150	4.87	3.24
Syzygium	Methanol	150	3.23	2.15
cumini	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.	5	67.82±0.00^{b}	42.91±1.98^{a}	57.10±2.37^{a,b}
µg/ml	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}	Methanol	5.18	6.93	78.31
values	Aqueous	23.22	69.97	7.19
µg/ml	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^{i}	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.

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