

**“IN VITRO EVALUATION OF ANTICOCCIDIAL
PROPERTIES OF SELECTED EDIBLE NUT BY-PRODUCTS
ON *EIMERIA* OOCYSTS OF POULTRY”**

By

Dr. MOGILI SUDHA RANI

B.V.Sc & A.H.

ID. No. GVM/18-033

Thesis submitted to the

SRI VENKATESWARA VETERINARY UNIVERSITY

*In partial fulfillment of the requirements
for the award of the degree of*

MASTER OF VETERINARY SCIENCE

In the Faculty of Veterinary Science

(VETERINARY PARASITOLOGY)



**DEPARTMENT OF VETERINARY PARASITOLOGY
NTR COLLEGE OF VETERINARY SCIENCE, GANNAVARAM - 521 102
SRI VENKATESWARA VETERINARY UNIVERSITY
TIRUPATI - 517 502 (A.P), INDIA.**

June, 2022

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CERTIFICATE

Ms. MOGILI SUDHA RANI, GVM/2018-033 *has satisfactorily prosecuted the course of research and that the thesis entitled “IN VITRO EVALUATION OF ANTICOCCIDIAL PROPERTIES OF SELECTED EDIBLE NUT BY-PRODUCTS ON EIMERIA OOCYSTS OF POULTRY” submitted is the result of original research work and is of the sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any University.*

Date:

(Dr. Ch. Jyothi Sree)
Major Advisor
Associate Professor
Department of Veterinary Parasitology
NTR College of Veterinary Science
Gannavaram-521 102

CERTIFICATE

This is to certify that the thesis entitled “IN VITRO EVALUATION OF ANTICOCCIDIAL PROPERTIES OF SELECTED EDIBLE NUT BY-PRODUCTS ON EIMERIA OOCYSTS OF POULTRY” submitted in partial fulfillment of the requirements for the degree of MASTER OF VETERINARY SCIENCE of the Sri Venkateswara Veterinary University, Tirupati, is a record of bonafide research work carried out by Ms. MOGILI SUDHA RANI, GVM/2018-033 under my guidance and supervision. The subject of the thesis has been approved by the Student’s Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigations have been duly acknowledged by the author of the thesis.

(Dr. Ch. Jyothi Sree)
*Chairman of the Advisory
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Thesis approved by the Student’s Advisory Committee

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DECLARATION

I, **MOGILI SUDHA RANI** hereby declare that the thesis entitled “**IN VITRO EVALUATION OF ANTICOCCIDIAL PROPERTIES OF SELECTED EDIBLE NUT BY-PRODUCTS ON EIMERIA OOCYSTS OF POULTRY**” submitted to Sri Venkateswara Veterinary University, Tirupati for the degree of **MASTER OF VETERINARY SCIENCE** is the result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

Date:

(MOGILI SUDHA RANI)

Place: Gannavaram

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Dr. M.Sudha Rani 

LIST OF SYMBOLS AND ABBREVIATIONS

%	: Per cent or Percentage
±	: Plus or minus
>	: Greater than
<	: Lesser than
°C	: degrees Celsius
µm	: Micrometre
µL	: Microlitre
mm	: Millimetre
mg/ml	: Milligram per milliliter
min	: Minutes
SLN	: Salinomycin
DMSO	: Dimethyl sulfoxide
OPG	: Oocysts per gram
<i>et al.</i>	: and others
etc.	: and other similar things
g	: Grams
h	: Hours

H_2SO_4	: Sulphuric acid
DOT	: 3,5-Dinitro-o-Toulamide
$\text{K}_2\text{Cr}_2\text{O}_7$: Potassium Di Chromate
ED_{50}	: Effective Dose 50
ml	: Millilitre
N	: Normality
PBS	: Phosphate Buffer Saline
Ph	: Potential of hydrogen
RPM	: Revolutions per minute
S	: Seconds
TLC	: Thin Layer Chromatography
UV	: Ultraviolet
V/V	: Volume / volume
SIA	: Sporulation Inhibition Assay
W/V	: Weight / volume
W/W	: Weight / weight

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ABSTRACT

The present study was conducted to evaluate the effect of methanolic extracts from cashew (*Anacardium occidentale*) nut peel, green coconut (*Cocos nucifera*) endocarp, almond (*Terminalia catappa*) hulls and partially developed groundnut (*Arachis hypogea*) kernel on sporulation and morphology of oocysts of *Eimeria* species of poultry. Sporulation inhibition bioassay was used to evaluate the activity of four extracts on the sporulation of coccidian oocysts. In this assay, unsporulated oocysts were exposed to five (300, 100, 30, 10, and 3 mg/ml) concentrations of each extract in distilled water in Petri dishes and cell-culture plates while salinomycin, ethopabate, formalin and potassium dichromate solution served as control groups. The Petri dishes and cell-culture plates were partially covered to allow the passage of oxygen and incubated at 25-29°C for 48 h, providing 80% humidity. The sporulation of the oocyst was confirmed by examining sporocysts under microscope at 40x at the end of incubation. The methalolic

extracts of *A. occidentale* peel and *T. catappa* hulls showed significant ($P < 0.01$) inhibitory effect (88.58 ± 0.39 and $79.24 \pm 0.83\%$, respectively) at highest concentration of 300 mg/ml on sporulation of *Eimeria* oocysts in comparison with negative control, with reference drugs *i.e.*, salinomycin, ethopabate and formalin (10%) (92.91 ± 1.80 , 84.64 ± 2.73 and $98.03 \pm 1.04\%$, respectively) being more effective than the plant extracts. The inhibition of sporulation was not dose dependent for *A. occidentale* nut peel and *T. catappa* hulls extracts. At highest concentration (300 mg/ml) the rate of sporulation inhibition by *C. nucifera* endocarp and partially developed *A. hypogea* kernels was 8.07 ± 2.34 and 1.43 ± 0.75 per cent respectively, which was imperceptible in comparison with the reference drugs. Extracts of *A. occidentale* peel induced 50% inhibition at lower concentration ($1.099 \mu\text{g/ml}$) compared to *T. catappa* extract ($7.627 \mu\text{g/ml}$). All four extracts also damaged the normal morphology and shape of the *Eimeria* oocysts. Thin Layer Chromatography analysis and qualitative phytochemical screening of extracts revealed presence of flavonoids, tannins, saponins, carbohydrates, triterpenoids and alkaloids that might be responsible for the anticoccidial effects noticed. In conclusion, this *in vitro* study suggests that *A. occidentale* peel and *T. catappa* hulls have promising anticoccidial effects.

CHAPTER – I

INTRODUCTION

India is the third largest producer of eggs and fifth largest producer of poultry meat in the world (Rajendra Prasad, 2022). Over the period of four decades, India's chicken business has evolved from a simple backyard occupation to a large commercial agri-based industry, and is the fastest growing segments of the agricultural sector with around eight per cent growth rate per annum (Chatterjee and Rajkumar, 2015). In 2018, the broiler and egg market in India was valued at INR 1,750 billion. The market is expected to increase at a CAGR of 16.2 per cent between 2019 and 2024, reaching INR 4,340 billion (Singh, 2019).

Coccidiosis, caused by *Eimeria*, an intestinal protozoan parasite is one of the most important and common health problems in the poultry industry. *Eimeria* is a genus of apicomplexan parasites that includes various species responsible for the development of coccidiosis. All species of *Eimeria* invade the intestinal mucosa; however, seven species are considered of economic importance, due to their proven pathogenicity such as, *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella* (Taylor *et al.*, 2016). The infection with two or more species of *Eimeria* is common in field, in which each species causes an independent and recognizable intestinal injury, apart from the other species. Coccidia are transmitted to the host orally through the contamination of food and water with sporulated oocysts of *Eimeria* spp. The global economic losses due to coccidiosis was estimated at over \$3 billion annually (Dalloul and Lillehoj, 2006; Shivaramaiah *et al.*, 2014).

Generally, coccidiosis control programmes mostly depend on chemotherapy and immunoprophylaxis. Anticoccidial drugs, coccidiostats added to the feed constitute a good preventative measure. A continuous in-feed low concentration of anticoccidial drugs to control *Eimeria* was reported early in the 1930s and mid-20th century (Grumbles

et al., 1948; Levine, 1940) and it was possible to control coccidiosis by the continuous inclusion of a low level of a drug in the feed of chickens. Anticoccidial drugs were used effectively over the past 50 years. Their use resulted in a rapid growth of poultry industry and affordable poultry products to the consumers. However, the use of suboptimal levels of the anticoccidial drugs increased the probability of selecting drug-resistant strains (Chapman, 1984). Shortly after introduction of sulphaquinoxaline and nitrofurazone, resistance had been reported (Cuckler and Malanga, 1955; Waletzky *et al.*, 1954). Since then, number of reports regarding anticoccidial drugs resistance including ionophores was reported (Chapman, 1997; Peek and Landman, 2003) due to widespread and prolonged use of coccidiostats.

The increasing resistance to *Eimeria* species and residual effects of drugs in meat and eggs has stimulated the efforts to search for alternative control measures (Abbas, 2012; Kim *et al.*, 2013; Ahmad *et al.*, 2016), the holistic approach by the application of herbal products for control of coccidiosis. Anticoccidial vaccines developed mainly for the replacement of the practice of incorporating coccidiostats in the feed, are not commercially available in different parts of world, including India. Moreover, vaccines, on the other hand might cause severe haemorrhagic reactions or malabsorptive coccidiosis, which can negatively impact flock performance (Chapman *et al.*, 2002; Shirley *et al.*, 2005). Thus, natural products, e.g. plants, extracts and their combinations, instead of synthetic drugs are believed to have an important role to play in the near future because they are usually residue-free and well accepted by consumers (Orengo *et al.*, 2012). In addition, plants and or their extracts as natural feed additives are considered to be one of the most promising alternatives to anticoccidials because of their natural stimulation of the immune system, increased growth performance and/or anticoccidial effects. In the last decade plant extracts were widely investigated, used for controlling avian coccidiosis and improving poultry performance worldwide (Abbas *et al.*, 2012;

Bozkurt *et al.*, 2014; Habibi *et al.*, 2014; Ahad *et al.*, 2017; Shekhar *et al.*, 2018; Adhikari *et al.*, 2020). For example, a 3% content of dried *Artemisia annua* leaves in the feed of broilers 3 weeks before subjecting the broilers to a coccidial challenge reduced oocyst excretion by 60–70% (De Almeida *et al.*, 2012). In Pakistan, smallholder farmers add turmeric powder to the feed to control coccidiosis in broilers (Abbas, 2012). Due to an increasing demand for natural products to control coccidiosis, this study was undertaken to evaluate the effect of four different herbal (cashew nut peel, green coconut endocarp, almonds hulls and partially developed ground nut kernel) extracts, which are known for their antioxidant and anti-inflammatory properties, on sporulation of coccidian oocysts with the following objectives.

1. To conduct qualitative phytochemical screening of the cashew nut peel, endocarp of green coconut, almond hulls and partially developed ground nut kernel
2. To determine the *in vitro* inhibitory effects of these extracts on sporulation of *Eimeria* oocysts of poultry

CHAPTER – II

REVIEW OF LITERATURE

Every year more than 40 billion chicken are raised to meet the protein source to humans in the form of meat and eggs (Quiroz-Castaneda and Dant an-Gonzalez, 2015). Globally, coccidiosis in chicken is an economically important parasitic disease and is a major constraint to the poultry industry in the form of reduced feed utilization, growth, and egg production, and sometimes death especially in broiler chicken (Abbas, 2012; Abbas *et al.*, 2015). Avian coccidiosis is caused by seven species of *Eimeria* (Gilbert *et al.*, 2011), an intestinal protozoan parasite, such as *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella* (Taylor *et al.*, 2016). In general, avian coccidiosis is controlled using anticoccidial drugs in feed or in water and or by vaccination. Though anticoccidial vaccines are developed, are not commercially available in different parts of world, including India. Thus, control was based exclusively on chemotherapy. Ionophorous antibiotics have been popularly used in the treatment of avian coccidiosis. But, the widespread use and abuse of anticoccidial drugs has resulted in development of resistance in *Eimeria* species (Shekhar *et al.*, 2018), even in spite of drug shuttle programmes and rotation programmes. In addition to drug resistant species, residual effects of drugs in chicken and public demand for residue-free meat and eggs, forced to look into alternative control strategies against avian coccidiosis (Molan *et al.*, 2009).

Use of natural compounds against coccidiosis is one of such alternative strategies. Natural compounds extracted from plants or produced by microorganisms are becoming more popular and can be utilised as anticoccidial feed additives in chicken. Some of these compounds are antioxidants that damage the parasite thus preventing the infection. They also promote antimicrobial immune responses to maintain chicken health haemostasis (Muthamilselvan *et al.*, 2016). Herbal anticoccidials have opened up new possibilities and are proven to be viable alternatives to conventional treatments, particularly in low-

income countries (Zaman *et al.*, 2015). The available literature on herbal remedies for coccidiosis control in chicken from different parts of the world is reviewed here under.

2.1. Importance of selected medicinal plants

2.1.1. *Anacardium occidentale* (Cashew):

The cashew tree is a tropical, evergreen, perennial plant that is native to tropical America (Iqbal *et al.*, 2021) and has been naturalised and cultivated in India, notably in coastal places such as Srikakulam, where the plant of interest is collected. The cashew nut, the tree's seed, is commonly consumed and the tree's leaves are known to have antioxidant and antibacterial properties (Chermahini *et al.*, 2011). The juice of the cashew apple has antiscorbutic properties and is used as a diuretic in the treatment of renal disease, cholera, pharyngitis and chronic dysentery. Cashew apple brandy is used as a pain reliever and nut oil is applied to broken heels. The bark and leaves are used to treat parasite infection, diarrhoea, wounds, rashes and pyrexia (Iqbal *et al.*, 2021). The root is a purgative and the tar from the bark is used as a counter irritant for external application in leprosy, ringworm and ostinate ulcers, while the stem has anti-inflammatory properties (Mota *et al.*, 1985). Eczema, psoriasis, scrofula, dyspepsia, venereal illness, impotency, bronchitis, cough, colic, leishmaniasis and syphilis are all treated with the leaves (Franca *et al.*, 1993).

2.1.2. *Terminalia catappa* (Indian almond):

Almond is a deciduous tree native to Iran and its surrounding Central Asian nations. It is a member of the Rosaceae family and is widely cultivated in a variety of conditions (Kahlaoui *et al.*, 2019). Memory loss, headaches, sleeplessness, and hepatosplenic disorders are treated with almond (Abdullah, 2017). Almond is used in confectioneries, cakes, and its butter as a substitute for dairy butter, and its milk as a substitute for cow's milk for those who have lactose intolerance. Almond oil is used to treat brain dryness, kidney infections, and bladder stones, while the almond kernel is used as a laxative, anti-tussive, and cerebrotonic. The roots are used to cure pityriasis. Almond oil

and honey are used to cure wounds, while almond oil mixed with hot water helps sore throats. It improves the strength and lustre of teeth (Mushtaq *et al.*, 2015). Almond paste with wheat starch and mentha is used to stop bleeding and treat chronic coughs and pneumonia. It improves colic and constipation, and it's also used to treat peptic ulcers. Almond powder with kateera is beneficial for dry cough (Abdullah *et al.*, 2017).

2.1.3. *Cocos nucifera* (Coconut):

Cocos nucifera is an arborescent, perennial monocot tree of the Arecaceae family native to Southeast Asia and Malasia, but widely dispersed throughout the world's tropics and subtropics (Kannaian *et al.*, 2020). *Cocos nucifera* shell fibres are utilised in diarrhoea, antipyretics, renal inflammation, dermatitis, wounds and traumas, as well as asthma and diabetes. Diarrhoea and stomach problems are treated using leaves and roots. Antipyretic, antidiarrheal, hair loss prevention, wound healing, oral contraceptive and aphrodisiac are all uses for oil, pulp and milk. Fever and malaria are treated with the white surface of the coca plant. It has analgesic, antimicrobial, antioxidant, antiosteoporosis, anti-diabetic, anti-neoplastic, anthelmintic, antihypertensive and anti-malarial properties (Akter *et al.*, 2020). Coconut water was served as an emergency short-term intravenous hydration fluid during World War II (Victor, 2013).

2.1.4. *Arachis hypogea* (Groundnut):

Peanut, often known as groundnut, is an annual herbaceous legume native to northern Nigeria and Cameroon that belongs to the Fabaceae family. Cystitis is treated using peanut oil, which also serves as a solvent for intramuscular injections (Geetha *et al.*, 2013). Peanut skins are used to treat bronchitis and chronic bleeding. Groundnut extracts have been used to help diabetic people regulate their blood sugar levels, as well as to lower cholesterol, improve weight loss and prevent cardiovascular disease and cancer. Leaves have astringent action and used therapeutically against abdominal pain, bronchitis, constipation and flatulence. Peanuts are having anti-inflammatory, antitumor, antifungal, antibacterial, antiproliferative, antioxidant, hypoglycaemic, hypolipidemic activities

and antiplatelet aggregation (Lopes *et al.*, 2011). Resveratrol, an antioxidant found in root, helps to prevent cancer, decrease cholesterol and improve heart health. Biodiesel fuel is made by combining root oil with other food oils and a low-grade petroleum blend. Vanillin found in peanut hulls has an effect on human cancer cells (Geetha *et al.*, 2013).

Plants have medical value because they contain a chemical molecule that has a physiological effect on the host body. Secondary metabolites protect plants from pathogens and are divided into three categories: phenolic compounds (phenols, phenolic acids, flavonoids, coumarins, tannins, etc.); terpenes (carotenes, steroids, saponins, triterpenes, etc.); and alkaloids (nitrogenous compounds) (Sarojini *et al.*, 2012; Abhishek and Thakur, 2012). Most tree nuts are high in tocopherols, total phenols and a wide spectrum of flavonoids and proanthocyanidines, which have antioxidant characteristics that may add to the overall health benefits of diet (Bolling *et al.*, 2010). Phenolic molecules are thought to be the primary cause of pharmacological activity (Oliveira and Pintado, 2015).

2.2. Anticoccidial activity of medicinal plants:

Youn and Noh (2001) conducted an *in vivo* experiment to determine the anticoccidial effect of 15 herbs viz., *Gleditsia japonica*, *Melia azedarach*, *Torilis japonica*, *Artemisia annua*, *A. asiatica*, *Quisqualis indica*, *Bupleurum chinese*, *Inula helenium*, *Pulsatilla koreana*, *Sophora flavescens*, *S. japonica*, *Torreya nucifera*, *Zuccarini*, *Ulmus macrocarpa* and *Sinomenium acutum* against the *E. tenella* infection in broiler chicks. The herb extracts were supplied in drinking water 5 days after challenge. The data of the survival rates, bloody diarrhoea symptoms, lesion scores, body weight gains and oocyst excretions indicated that the extract of *S. flavescens* was the most effective. Whereas, *Pulsatilla koreana*, *S. acutum*, *U. macrocarpa* and *Q. indica* were also effective.

Arab *et al.* (2006) used high-performance liquid chromatography to detect the presence of artemisinin in *Artemisia sieberi* and to test the anticoccidial properties of plant extract in broiler chicks challenged with various *Eimeria* species (*E. tenella*, *E. acervulina* and *E.*

maxima). Artemisinin extracts at doses of 1 and 2.5 mg/kg body weight per day orally, reduced the severity of *E. tenella* and *E. acervulina* infection, but not *E. maxima*.

Kurkure *et al.* (2006) evaluated the anticoccidial efficacy of “Coxynil” (polyherbal preparation) (*Allium sativum* 15%, *Cinnamomum camphora* 15%, *Elephantopus scaber* 15%, *Valeriana wallichii* DC 15%, Sulphur dioxide (Gandak) 25% and NaCl 15%) in broilers. Compared to untreated birds, the Coxynil-treated birds had no second generation schizonts in the caeca and had a greater body weight indicating anticoccidial property of Coxynil.

Jang *et al.* (2007) investigated the anticoccidial efficacy of green tea-based diets after oral challenge with *E. maxima* oocysts at the age of five weeks. Compared to the control group, green tea fed hens produced considerably less faecal oocysts ($p < 0.05$) without showing improvement in body weight.

The effect of aqueous extract of aloe gel (10% w/v) on growth performance and faecal coccidial oocysts count following natural progression of coccidiosis in broilers reared at Peshawar was studied by (Durrani *et al.*, 2008). One hundred and sixty, day old chicks were randomly allotted to four different groups, designated as A, B, C and D in an open sided house. Group A, B and C received aqueous extract of aloe gel (10% w/v) @ of 5, 10 and 15 ml/litre of water and group D was kept control for 35 days. On day-21 of the experiment, all the chicks were inoculated with oocysts of different species of *Eimeria* and in addition, oocysts were seeded in the litter. Water and feed intake, abdominal fat deposition and weight of breast, thigh gizzard, heart and liver of broilers given aloe extract in drinking water was not different among groups. Significantly higher body weight gain, dressed weight and lower feed conversion ratio was observed for broilers in group B *i.e.*, treated @ of 10 ml/litre. Significantly higher mortality (10%) was found in control group.

Khan *et al.* (2008) designed a study to compare the efficacy of some herbal and

homeopathic preparations against coccidiosis on the basis of weight gain, feed conversion ratio, oocyst count and mortality rate. A total of 240, day-old broiler chicks reared under standard management practices were randomly divided into six groups (A to F) on 22nd day of age. The chicks of all the groups except group F were inoculated orally with sporulated oocysts and treated with *Poligonum bistorta* (Anjbar), *Agele marmelos* (Bael fruit), Merc sol. (*Mercurius solubilis*) and Darvisul liquid. *Agele marmelos* and Darvisul liquid showed better results in terms of weight gain, feed consumption, oocyst count as compared with *P. bistorta* (Anjbar) and *Mercurius solubilis*.

Naidoo *et al.* (2008) conducted an *in vivo* experiment to assess the anticoccidial activity of four plant extracts (*Combretum woodii*, *Tulbaghia violacea*, *Vitis vinifera* and *Artemisia afra*) along with toltrazuril as a positive control. Treatment with *Tulbaghia violacea* (15 mg/kg), *Vitis vinifera* (75 mg/kg) and *Artemisia aftu* (150 mg/kg) produced feed conversion ratios similar to toltrazuril and greater than the untreated control whereas, *Combretium wedi* (160 mg/kg) proved to be extremely toxic to the birds and *T. violacea* showed significant oocyst reduction.

The effect of mixed oil extract of *Artemisia annbozkuetua*, *Hyssopus officinalis* and *Pimpinella anisum*, called Polioel 3, on sporulation, morphological damages, population structure and virulence of *Eimeria* oocysts was checked by (Titilincu *et al.*, 2008) using different dilution, namely 1:25, 1:50, 1:100, 1:500 and 1:1000 and two controls (2.5% potassium dichromate and 0.5% Tween 80). Results revealed the lowest rate of sporulation at a dilution of 1:100 (64.4±4.77%) and also the highest number of damaged oocysts (33.4±7.76%), the difference being significant in comparison with the controls.

Chandrakesan *et al.* (2009) conducted a study to know the anticoccidial efficacy of an herbal complex consisting of *Solanum nigrum* (35%), *Aloe vera* (15%), *Moringa indica* (35%) and *Mentha arvensis* (15%) in broilers. The birds were treated with herbal compound at dose

rates of 5 and 10% for 7 days after an *Eimeria* challenge infection. Salinomycin mixed feed and uninfected unmedicated birds used as control groups to know the effectivity of herbal complex. The birds that received 10% herbal complex exhibited better body mass gain between the 4th and 5th weeks (344.34 ± 59.81 gm), superior feed conversion ratio (1.77 ± 0.43) and moderate caecal length (11.5 ± 1.19 cm) with no significant difference in the oocyst output between treated and control groups.

Molan *et al.* (2009) undertook series of experiments to determine the effect of water extracts from pine bark (*Pinus radiata*) on the inhibition of the sporulation of oocysts of three species of avian coccidia. Tubes containing coccidian oocysts isolated from droppings of coccidia-infected chickens were randomly assigned to 0, 250, 500 and 1000 µg/ml pine bark extract (PBE). The tubes were incubated at 25-29 °C for 48 h depending on the species of *Eimeria*. Sporulation inhibition bioassay was used to evaluate the activity of PBE on the sporulation of coccidian oocysts. The oocysts were gently aerated with an air pump away from sun light. The results showed that water-soluble extracts from pine bark containing 35% condensed tannins have anticoccidial activity as evidenced by their ability to decrease significantly the sporulation of the oocysts of three species of *Eimeria*, namely *E. tenella*, *E. maxima* and *E. acervulina* under laboratory conditions. Incubation of unsporulated oocysts of these parasites in water containing 500 µg/ml PBE resulted in inhibition of sporulation of these oocysts by about 28-84% relative to the oocysts in the control incubations. In addition, up to 12% of *E. maxima* oocysts exposed to 500-1000 µg pine bark/ml were containing abnormal sporocysts in terms of size, number and shape.

Maslinic acid (2- α , 3- β -dihydroxiolean-12-en-28-oic acid), found in the leaves and fruit of the olive tree (*Olea europaea*), was documented as a natural coccidiostatic against *E. tenella* (De Pablos *et al.*, 2010) as considerable increase in weight was found in the chicks treated with maslinic acid compared with those in the

control group. Histopathological studies of the caecum at 120 h post-infection showed that the infection rate decreased significantly in chicks treated with maslinic acid.

Anosa and Okoro (2011) designed a study to evaluate the anticoccidial activity of the methanolic extract of *Musa paradisiaca* root in chickens. The chickens were divided into six groups of 12 chickens each. Each chicken in five groups was infected with 8,000 infective coccidia (*E. tenella*) oocysts at day 28 of age while one group served as uninfected control. At day 7 post-infection, two chicken in each group were sacrificed for postmortem examination to confirm coccidiosis. Also at day 7 post-infection, each chicken in four infected groups was given graded doses (250, 500 and 1,000 mg/kg b.wt) of the extract or amprolium (conventional drug). Two groups (an infected and uninfected group) did not receive treatment. Parameters used to assess progress of infection and response to treatment included clinical signs typical of coccidiosis, oocyst count per gram of faeces (OPG) and packed cell volume (PCV). Treatment of previously infected chickens with *M. paradisiaca* root extract resulted in a progressive decrease in severity of observed clinical signs, marked reductions in OPG and a gradual increase in PCV. In each case, the changes were dose dependent. There was no significant difference in mean OPG and mean PCV of the extract (at 1,000 mg/kg b.wt) and amprolium-treated groups at termination of the study (at day 50 of age). The results of this study demonstrated that the extract has anticoccidial activity in a dose-dependent manner and at a dosage of 1,000 mg/kg b.wt had similar efficacy with amprolium in the treatment of chicken coccidiosis.

Anticoccidial effects of curcumin (diferuloylmethane), a natural polyphenolic compound abundant in the rhizome of the perennial herb turmeric (*Curcuma longa*) were evaluated on *E. tenella* sporozoites, including morphological alterations, sporozoite viability and infectivity to Madin-Darby bovine kidney (MDBK) cells (Khalafalla *et al.*, 2011). Curcumin at concentrations of 25, 50, 100, 200 and 400 μm showed considerable effects on sporozoite morphology and viability in a dose-dependent manner after

incubation over 3, 6, 18 and 24 h while lower curcumin concentrations (6.25 and 12.5 μm) were not effective. In comparison to the untreated control, sporozoite infectivity was reduced at curcumin concentrations of 100 and 200 μm by 41.6% and 72.8%, respectively. Negative effects of curcumin on MDBK cells were not seen at these concentrations; however, curcumin at concentrations of 1,800, 600 and 400 μm was toxic to MDBK cells and affected cell proliferation.

Michels *et al.* (2011) evaluated the anticoccidial efficacy of a product containing coumestans from *Eclipta alba in vitro*. Male Cobb broilers (240) were assigned to four experimental groups (T1 to T4). The T1 group received standard feed (negative control); T2 was treated with standard feed supplemented with 66 ppm of salinomycin (positive control); groups T3 and T4 had standard feed supplemented with the ethyl acetate fraction from methanolic extract of *E. alba* aerial parts, which contains the coumestans WL and DWL (120 and 180 ppm, respectively). The chicken broilers were individually infected with 2×10^4 oocysts of *E. tenella* when they were 14 days old and were monitored weekly to evaluate the weight gain and food conversion ratio. Counting of coccidial oocyst in chicken faeces was assessed from random samples, from 7-14 days after the infection. The group treated with coumestans from *E.alba* presented an average weight gain and food conversion ratio higher than the negative control group and similar to the mean value of the positive control group. Coumestan-treated groups showed a significant decrease in the oocyst counting since the 21th day of life. Overall, results validated the use of a phytotherapy containing *E.alba* coumestans at a dose of 120 ppm as a therapeutic or prophylactic agent against avian coccidiosis.

The aqueous stem bark extracts of *Khaya senegalensis*, *Butyrospermum paradoxum* and *Anona senegalensis* were evaluated for toxicity and antococcidial effects in *in vitro* and *in vivo* studies (Nwosu *et al.*, 2011). Phytochemical analysis revealed that tannins, terpenes, anthraquinones, phlobotannins, alkaloids, cardiac glycosides and steroids were

present in various concentrations in some of the extracts. Extracts of *K. senegalensis* had no *in vitro* anti-sporulation effect while those of *B. paradoxum* and *A. senegalensis* produced significant effects that were concentration dependent and comparable to amprolium at 0.6 and 1.2 mg ml⁻¹ concentrations. Oral treatment of pullet chicks infected with 120,000 sporulated *Eimeria* oocysts/chick with either the extracts or amprolium significantly reduced or eliminated faecal oocyst output and improved packed cell volume and live weight of the birds. These effects were highest with *A. senegalensis* and *K. senegalensis*. Further, *B. paradoxum* was more toxic in toxicological studies, which precludes its possible use as an anticoccidial agent.

Séverin *et al.* (2012) conducted a study to determine the effect the aqueous extract of *Thonningia sanguinea* on cell invasive property of *E. tenella* and *E. necatrix* sporozoites. Results revealed that the extracts at concentration above 2.5 mg/ml significantly ($p < 0.05$) inhibited the cell invasion by sporozoites of both the species.

Zaman *et al.* (2012) evaluated the anticoccidial effect of different concentrations of the herbal complex of 4 plants (leaves of *Azadirachta indica* and *Nicotiana tabacum*, flowers of *Calotropis procera* and seeds of *Trachyspermum ammi*) in broiler chickens in comparison with commercial drug amprolium. Three concentrations (2 g, 4 g and 6 g) of herbal complex were given to the experimental groups once a day and amprolium (at the dose rate of 125 ppm) was given orally in drinking water from the 14th to the 21st days of age. Among the herbal complex medicated groups, the maximum anticoccidial effect was seen in the group medicated with 6 g herbal complex followed by 4 g and 2 g herbal complex. Treatment with 6 g of the herbal complex significantly reduced the negative performance and pathogenic effects associated with *E. tenella* challenge at a level that was comparable to amprolium.

Zhang *et al.* (2012) conducted a study on broiler birds to evaluate the anticoccidial efficacy of an extract of Chinese traditional herb *Dichroa febrifuga*. One hundred broiler

birds were assigned to five equal groups. All birds in groups 1–4 were orally infected with 1.5×10^4 *E. tenella* sporulated oocysts and birds in groups 1, 2 and 3 were medicated with 20, 40 mg extract/kg feed and 2 mg diclazuril/kg feed, respectively. The results showed that *D. febrifuga* extract was effective against *Eimeria* infection; especially 20 mg *D. febrifuga* extract/kg feed could significantly increase body weight gains and reduce bloody diarrhoea, lesion score and oocyst excretion in comparison to infected-unmedicated control group.

Burt *et al.* (2013) evaluated the effect of betaine, carvacrol, curcumin and *Echinacea purpurea* extract (EP) phytochemicals on *E. tenella* sporozoite invasion *in vitro* on the basis that they reduce the virulence of *Eimeria* spp. and/or provide immune modulatory benefits to host cells. Madin-Darby bovine kidney (MDBK) cells were covered by medium containing phytochemicals at the highest concentration which was non-toxic to the cells. Salinomycin 50 µg/ml was used as positive control and a negative control was medium only. *Eimeria tenella* (Houghton strain) sporozoites were added to wells and after incubation for 2, 4 or 20 h at 37 °C, cells were fixed and stained with hematoxylin-eosin. Ten evenly spaced fields per well were photographed and the percentage of cells invaded by sporozoites was calculated and normalized to the control. Results showed that invasion of MDBK epithelial cells by *E. tenella* sporozoites was inhibited in the presence of carvacrol, curcumin or EP and enhanced by betaine.

The effects of dietary supplementation with an organic extract of *Curcuma longa* on systemic and local immune responses to experimental *E. maxima* and *E. tenella* infections were evaluated in commercial broiler chickens (Kim *et al.*, 2013). Dietary supplementation with *C. longa* enhanced coccidiosis resistance as demonstrated by increased body weight gains, reduced faecal oocyst shedding, and decreased gut lesions compared with infected birds fed a non-supplemented control diet. The chickens fed *C. longa*-supplemented diet showed enhanced systemic humoral immunity, as assessed by greater levels of serum

antibodies to an *Eimeria* microneme protein, MIC2, and enhanced cellular immunity, as measured by concanavalin A-induced spleen cell proliferation, compared with controls. Results suggested that dietary *C. longa* could be used to attenuate *Eimeria*-induced, inflammation-mediated gut damage in commercial poultry production.

The efficacies of five dietary supplements were screened on performance indices, faecal oocyst excretion, lesion score and intestinal tract measurements in healthy and experimentally infected with oocysts of mixed *Eimeria* spp. birds by (Bozkurt *et al.*, 2014). Birds were fed five diets containing preparations of 60 mg/kg of anticoccidial salinomycin (SAL), 1 g/kg of multienzyme (ENZ), 1 g/kg of probiotic (PRO), 1 g/kg of prebiotic (PRE) and 40 mg/kg of an herbal essential oil mixture (EOM). Body weight gain and feed conversion ratio (FCR) showed significant improvement in the infected animals, which indicated that dietary supplemental regimens with SAL, ENZ, PRO and PRE initiated in 1-day old chicks reduced adverse effects after challenge with coccidiosis; however, chicks that were administered EOM failed to show such improvement. Uninfected chickens showed improvement in FCR with supplements SAL, PRE and EOM. In the infected group, all of the supplements reduced the severity of coccidiosis lesions ($P < 0.01$) induced by mixed *Eimeria* spp. through the middle and lower regions of the small intestines, whereas, supplementation with SAL or EOM alone was effective ($P < 0.01$) in reducing oocyst excretion compared with the control treatment.

El-Khtam *et al.* (2014) evaluated the efficacy of turmeric (*Curcuma longa*) and garlic (*Allium sativum*) on *Eimeria* species in broilers. In the *in vitro* study, the efficacy of garlic was higher (up to 80%) than turmeric (up to 66.6%) at different concentrations (10, 5, 2.5, 1.25, 0.6, 0.3, 0.2 and 0.08 g powder /liter distilled water). In the *in vivo* study, clinical signs and lesion score were less severe in garlic supplemented groups compared with turmeric supplemented groups. Reduction of total oocyst count in garlic supplemented group more than turmeric supplemented group. It was concluded that garlic powder was more effective than turmeric powder in treatment and control of coccidiosis.

Habibi *et al.* (2014) evaluated the *in vitro* and *in vivo* anticoccidial effects of leaves of seven different herbs namely *Biarum bovei*, *Nectaroscordum tripedale*, *Dorema aucheri*, *Cichorium intybus*, *Prangos ferulaceae* and *Artemisia absinthium* in comparison with the diclazuril on *E. tenella*. In *in vitro* assay, *Prangos ferulaceae* and *Cichorium intybus* extracts showed the highest (29.69%) and lowest (8.51%) percentage of sporulation inhibition at the end of 72 hours, respectively. While in *in vivo* studies, *N. tripedale* extracts and diclazuril showed better results in terms of growth performance, lesion score, extent of bloody diarrhoea and oocyst count compared to other extracts.

Fatemi *et al.* (2015) compared the effect of different *Artemisia annua* extracts including petroleum ether (PE), ethanol 96° (E) and water (W) extracts on sporulation rate of mixed oocysts of *Eimeria acervulina*, *E. necatrix* and *E. tenella*. Sporulation inhibition assay was used to evaluate the activity of extracts. The results showed that the E and PE extracts inhibit sporulation in 2 and 5 ppt concentrations, but the W extract stimulates it in all concentrations. The proportions of oocyst inhibition relative to control were 31 % (5 ppt) and 29 % (2 ppt) for PE and 34 % (5 ppt) and 46 % (2 ppt) for E extract. Furthermore, many oocysts in PE and E groups were wrinkled and contained abnormal sporocysts. The proportions of sporulation stimulation relative to control were 22 % (5 ppt), 24 % (2 ppt) and 27 % (1 ppt) in W extract.

Abbas *et al.* (2015) evaluated the effect of aqueous methanolic extract from *Saccharum officinarum* (SOE) on the sporulation and morphology of oocysts of *Eimeria* species of poultry. Sporulation inhibition bioassay was used to evaluate the activity of SOE on the sporulation of coccidian oocysts. In this assay, unsporulated oocysts were exposed to six (10, 5, 2.5, 1.25, 0.625 and 0.31%) concentrations of *S. officinarum* at 25-29 °C for 48 h, while DMSO and potassium dichromate solution served as control groups. Results showed anticoccidial activity of SOE against all *Eimeria* species by inhibiting the sporulation of the oocysts under laboratory conditions. Inhibition of sporulation was

observed in dose dependent manner. *Saccharum officinarum* extract at higher dose also damaged the normal morphology and shape of oocysts of *Eimeria* species.

Arczewska-Włosek and Świątkiewicz (2015) reported that feed supplemented with herbal extract (1 g/kg feed) containing garlic (*Allium sativum*), sage (*Salvia officinalis*), echinacea (*Echinacea purpurea*), thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) showed increased growth performance and reduced the oocyst output similar to the group fed with coccidiostat.

Kostadinovic *et al.* (2015) evaluated the efficacy of anticoccidial activity of numerous natural products such as *Ageratum conyzoides* extract (billy goat weed), green tea, maslinic acid, extracts of *Polygonum bistorta* (Anjbar) and *Agele marmelos* (Bael), *Artemisia sieberi*, *A. absinthium*, the leaves of Neem (*Azadirachta indica*), *A. annua* and Aloa vera-based supplements.

Molan and Faraj (2015) used a sporulation inhibition bioassay to examine the effect of selenium-rich green tea extract in different concentrations (0%, 5% and 10%) on *Eimeria* species of chicken. The green tea extract dramatically reduced ($P < 0.0001$) the sporulation of *Eimeria tenella*, *E. maxima* and *E. acervulina* oocysts with inhibition ranging from 28 to 84 per cent as compared to control. The shapes and sizes of *Eimeria* sporocysts collected from incubations containing the tea extract were aberrant.

Effect of leaves of *Curcuma longa*, seeds of *Artemisia absinthium* and roots of *Saussurea lappa* was evaluated on sporulation of *E. tenella* under laboratory conditions (Zaman *et al.*, 2015). The oocysts used in this study were obtained by challenging the 14th day-old broiler chicks with 75,000 sporulated oocysts of *E. tenella*. *E. tenella* oocysts were exposed to various doses of the 70% aqueous-methanolic herbal extracts (0.244-500 $\mu\text{g ml}^{-1}$) at 27.5°C temperature with 60-80% humidity under continuous aeration. Inhibition of sporulation was criterion for evaluation of the efficacy. The herbal extracts exhibited graded dose response on sporulation of *E. tenella* as evident by their lethal

concentration (LC₅₀) values. LC₅₀ values of *C. longa*, *A. absinthium* and *S. lappa* were 173.4, 221.3 and 960.6 µg ml⁻¹, respectively. *Curcuma longa* demonstrated the highest inhibitory effects on sporulation of *E. tenella* oocysts.

Efficacy of *Bidens pilosa* (BP) against chicken coccidiosis was investigated by Chang *et al.* (2016) in an organic chicken farm. The feed supplemented with BP, at the dose of 0.025% of feed or more, significantly reduced *Eimeria* infection. This treatment increased body weight gain and reduced feed conversion ratio, leading to superior growth performance. It lowered morbidity/mortality rate, decreased oocysts per gram of feces and gut pathology and augmented the anticoccidial index.

The anti-coccidial activity of *Moringa Oleifera* powdered leaves for prevention and its 75% ethanolic extract for treatment of coccidiosis were studied in broiler chicken (El Banna *et al.*, 2016). Inclusion of *M. olifera* powder (0.5 and 1%) in broiler feed was efficacious and comparable with that of diclazuril (1ppm) in preventing symptoms of coccidiosis associated with experimental infection with mixed *Eimeria* species. The experimentally infected chicks with mixed *Eimeria* species induced severe coccidiosis in non-medicated chicks and led to high mortality. Addition of 200 and 400 ppm of *M. oleifera* ethanolic extract induced marked curative effect of coccidiosis manifested by reduced mortality per cent and improved postmortem lesion compared to chicks treated with the reference drug, toltrazuril (25ppm).

In vitro anticoccidial activity of the methanolic leaves extract of *Lannea schimperi* was evaluated by (Mikail *et al.*, 2016). *Eimeria tenella* isolated from infected chicks was used in this study. The extract was tested at concentrations of 25, 50 and 100 mg/ml on unsporulated and sporulated oocysts of *E. tenella*. Amprolium at concentration of 1 mg/ml was used as a positive control, while 2.5 % potassium dichromate was used as negative control. The data obtained in this study showed that the extracts possess anticoccidial activity against unsporulated and sporulated oocysts of *E. tenella* in a dose dependent manner. The extract at concentration of 100 mg/ml inhibited oocyst

sporulation (98%) and inhibited the viability of sporulated oocysts (97%) similar to that recorded by the standard drug amprolium after 72 hours of incubation at room temperature. The negative control recorded 4 and 2% efficacy for unsporulated and sporulated oocysts, respectively, with 96 and 98% sporulated oocysts.

Ahad *et al.* (2017) designed a study to see the anticoccidial effect of different solvent extracts of *Artemisia vestita*. A high dose of crude extracts (300 mg/kg body weight) was tested for possession of anticoccidial activity against experimentally induced coccidial infection in broiler chicken. Activity was measured in comparison to the reference drug amprolium on the basis of oocyst output reduction, mean weight gain of birds and feed conversion ratio. The crude methanolic extract (CME) of *A. vestita* showed the maximum anticoccidial effect as evident by oocyst output reduction (71.5 ± 12.2), weight gain of birds (1406.4 ± 12.2) and feed conversion ratio (1.58 ± 0.06), thereby affirming the presence of alcohol soluble active ingredients in the plant. The effect was dose dependent.

Juasook *et al.* (2017) conducted a study the effects of pineapple (*Ananas comosus*) peel and core extracts for controlling *in vitro* and *in vivo* chicken caecal coccidiosis. *In vitro* results showed that the number of sporulated *E. tenella* oocysts in the pineapple peel and core extract groups decreased significantly ($p < 0.05$). Correspondingly, histopathological study of the cecum showed that infection of *E. tenella* was greatly decreased in these groups. Pineapple crude extract inhibited sporulation and decreased the chance of *E. tenella* infection, but may be toxic by inducing caecal tissue inflammation.

Ademola *et al.* (2019) conducted a study to investigate the organic chemical compound present in the *Pleurotus ostreatus* and its anticoccidial activity. The extract contained saponins, flavonoids, anthraquinones and alkaloids, which were used to assess oocyst output inhibition, lesion score, faecal score, weight differences, haematological parameters and leucocyte differential counts. The acute toxicity investigation found that it was nontoxic at a level of 600 mg/kg. Reduced oocyst output was reported in treatment

groups with an ED₅₀ value of 448 mg/kg for the extract.

The anticoccidial, antioxidant and cytotoxic activities of *Psidium guajava* methanolic and ethyl acetate extracts was evaluated by (Cedric *et al.*, 2018) against the rabbit coccidiosis by *in vitro* tests (oocysticidal, ant sporozoidal and antioxidant assay). The test revealed highest oocysticidal efficacy as 88.67±2.52% at the concentration of 30 mg ml⁻¹. The highest viability inhibitory percentage was 97.00±1.73% at a concentration of 1000 µg ml⁻¹ of the methanolic extract against *E. intestinalis* sporozoites. The aqueous extracts also exhibited good anti-sporozoidal activities against *E. flavescens*, *E. stiedae*, *E. intestinalis*, and *E. magna* at 1000 µg ml⁻¹. Extracts also possessed strong antioxidant activities at (IC₅₀<20 µg ml⁻¹).

Dakpogan *et al.* (2018) evaluated anticoccidial activity of *Carica papaya* and *Venonia amygdalina* crude juice on *E. tenella* oocysts of poultry. The day old chicks were divided into four group and were experimentally infected sporulated oocysts. First and the second groups were treated with papaya and vernonia juice, for 5 days and the third with sulfadimidine and the fourth was unmedicated control. Oocyst sporulation was inhibited in first and the second groups by 59.31 and 40.78%. Further, the juice treated groups showed improvement in the survivability by 20% compared with the unmedicated control group.

Gadelhaq *et al.* (2018) carried out a study to determine the anticoccidial effect of two herbal extracts *Allium sativum* (garlic) and *Moringa olifera* and commercial disinfectants. The results revealed that the two herbal extracts and commercial disinfectants Dettol, TH4, Phenol, Virkon*S and Diclazuril 20% had no effect on the sporulation. Whereas, sodium hypochlorite showed sporulation inhibition of 49.67% and 70% ethanol and 10% formalin showed 100% sporulation inhibition.

López *et al.* (2019) carried out a study to evaluate the anticoccidial activity of methanolic extract of canary rue (*Ruta pinnata*), plant of the Canary Islands, Spain,

against *E. ninakohlyakimovae* by the *in vitro* tests (oocyst sporulation inhibition assay, sporozoite viability assay, sporozoite cell invasion assay, cytotoxicity assays). The study revealed that the effect of extract on oocyst sporulation inhibition was both time and concentration dependent and the concentrations >0.1 mg/ml affected the viability of the sporozoites and also their cell invasion capacity ($P < 0.001$). In addition, the extract at 3 mg/ml concentration inhibited sporulation of oocysts which was similar to that observed with positive control.

Shekhar *et al.* (2018) carried out an experimental study to evaluate the anticoccidial activity of aqueous and methanolic extract of *Melia azedarach* (Bakain) leaves in experimentally induced *E. tenella* infection in broilers. The results revealed that the absence of toxicity seen during the acute toxicity test even at highest dose. The birds treated with methanolic extract of *M. azedarach* leaves @1000 mg/kg body weight for 7 days showed a continuous decrease in severity of clinical signs, faecal score, average oocyst production, average oocyst index, average lesion score and increased per cent survival, per cent weight gain, performance index, per cent protection against lesion as well as improvements in haematological and biochemical parameters.

Udo and Abba (2018) conducted a study to evaluate the effect of aqueous and powder extract of *Allium sativum* and *Carica papaya* on unsporulated oocysts using *in vitro* sporulation inhibition assay. *Allium sativum* and *C. papaya* extracts were produced in distilled water (g/L) and (ml) at powder concentrations of (2.5 g, 5 g and 10 g) and aqueous concentrations of (2.5 g, 5 g, and 10 ml/litre of water). Unsporulated oocysts were exposed to each concentration for 1 hour, 18 hours and 48 hours. The results showed that 10 gm of all extracts inhibited sporulation activity after 1 hour, 18 hours and 48 hours.

A study conducted by (Abbas *et al.*, 2019) to determine the *in vitro* anticoccidial effect of *Trachyspermum ammi* (Ajwain) extract towards the four species of unsporulated oocysts (*E. tenella*, *E. brunetti*, *E. necatrix* and *E. mitis*) of chicken by sporulation

inhibition assay. Collected oocysts were subjected to six different concentrations (w/v) of 10, 5, 2.5, 1.25, 0.625 and 0.31% in 10% dimethyl sulphoxide solution (DMSO), while dimethyl sulphoxide and potassium dichromate solution were used as control groups. *Trachyspermum ammi* extract had an *in vitro* anticoccidial action, altering sporulation and damaging *Eimeria* oocysts in a dose-dependent way, as well as harming the morphology of oocysts in terms of shape, size and number of sporocysts.

Arlette *et al.* (2019) evaluated the *in vitro* anticoccidial activity of aqueous and ethanolic extracts of leaves of *Ageratum conyzoides* and *Vernonia amygdalina* on oocysts of *E. tenella*. The different concentrations (100, 50, 25 and 12.5 mg/ml) of *A. conyzoides* and *V. amygdalina* extracts were analysed with Tween 80 (3.5 %) and potassium dichromate (2.5%) as negative controls, whereas phenol 5% as positive control. The inhibition rate of ethanolic extracts were found to be concentration dependent and considerably greater at all concentrations compared to aqueous extracts after 48 hours of incubation. Independent comparisons revealed that at the concentration of 100 mg/ml or higher, the percentage inhibition of *A. conyzoides* was significantly higher ($P < 0.05$) than that of *V. amygdalina*.

Debbou-Iouknane *et al.* (2019) conducted a study to investigate the type of standard compound (quercetin and oleuropein) present in aqueous ethanol extract of olive pulp (*Olea europaea L var. Chemlal*) and to evaluate the anticoccidial activity on *Eimeria* species of broiler chicks. Phytochemical analysis revealed phenols, flavonoids and condensed tannins (Proanthocyanidins) in the extract and their antioxidant activity was determined using the ABTS•+ Radical, DPPH Radical, Ferric Reducing Power (FRAP) assay and High-Performance Liquid Chromatography–Mass Spectrometry (HPLC–ESI–MS) methods. The aqueous ethanol extract significantly inhibited the sporulation process at a concentrations of 0.023 to 0.371 mg/ml.

Abbas *et al.* (2020) designed a study to evaluate the effect of *Vitis vinifera* (grape

seed) extract on unsporulated *Eimeria* spp. oocysts using *in vitro* sporulation inhibition assay. The extracts were made in various concentrations (10, 5, 2.5, 1.25, 0.625, and 0.31%) in 10% dimethylsulfoxide (DMSO)), whereas DMSO and potassium dichromate solutions were used as controls. The findings revealed that *V. vinifera* (grape seed) extract had a dose-dependent inhibitory effect on *Eimeria* oocyst sporulation and also manifested damaged oocysts in terms of form, size and quantity of sporocysts.

Graded concentrations (200, 400 and 800 mg/kg) of the aqueous stem bark extract of *Khaya senegalensis* was evaluated by (Muraina *et al.*, 2020) for its therapeutic efficacy against experimentally induced coccidiosis in broiler chicken. The phytochemical analysis showed the presence of tannins, saponins, cardiac glycosides and steroids. There was significant reduction in oocyst count across the groups in a graded dose manner with 800 mg/kg being the most efficacious dose. There was also weight gain across the treatment groups with immuno-modulatory and erythropoetic activities observed. The haematology also showed a dose-dependent increase in red blood cells, haemoglobin and packed cell volume of the treatment groups. The extract had no significant difference ($p > 0.05$) on the white blood cells, but a slight decrease in the white blood cells and heterophil counts was observed at 400 mg/kg. Furthermore, the aspartate amino transaminase level showed a significant difference ($p < 0.05$). Conclusively, the aqueous extract of *K. senegalensis* was effective in the management of coccidiosis thus supporting its folkloric use.

Tamilvanan *et al.* (2020) designed a work to determine antsporulation of *Zingiber spectabile*, *Piper betle*, *Cissus quadrangularis*, *Costus pictus* and *Centella asiatica* extracts on cloacal isolates and *Eimeria* oocysts from Vanaraja birds. The results showed that methanolic extracts of *Zingiber spectabile* (root) and *Piper betle* (leaf) prevented sporulation at a much higher rate ($>80\%$) than control, with *Piper betle* inhibiting ($>80\%$) and *Zingiber spectabile* inhibiting ($>70\%$), respectively.

Aouadi *et al.* (2021) conducted a study to evaluate the *in vitro* anticoccidial activity of volatile oil of “*Rosmarinus officinalis*” and its major constituents namely – cineole, α -pinene, camphor by sporulation inhibition assay against small ruminants. The essential oil was found to be efficacious against *Eimeria* spp. oocysts in sheep, with an IC_{50} of 1.82 ug/ml.

Gonzaga *et al.* (2021) carried out a study to evaluate anticoccidial activity of *Coleus aromaticus* leaves extract in naturally infected goats as effective remedy for coccidia. Three concentrations (600, 800 and 1000 mg / kg b.wt) of *Coleus aromaticus* extract were tested for anticoccidial activity in comparison with a commercial coccidiostat, toltrazuril. The efficacies of the three concentrations were significant after 21 days of treatment, but slightly lower than toltrazuril ($p = 0.0394$). At 21 days post-treatment anticoccidial efficacy was 92.29% (600 mg/kg extract) for *C. aromaticus* when compared to the control toltrazuril (96.65%) indicating that the extract has potential effect as like as toltrazuril in reducing the oocyst counts of naturally-infected goats.

Oyeleke *et al.* (2021) designed a study to investigate the effect of aqueous extracts of *Petiveria alliacea* root and leaf on *Eimeria* oocysts and intestinal bacteria in poultry. The results showed that when hens were given @ 15, 30 and 45 g of *P. alliacea* extracts, *Eimeria* oocyst and intestinal bacteria counts were lower ($P = 0.0001$ and $P = 0.0028$, respectively).

Qaid *et al.* (2021) conducted an experimental *in vivo* study to evaluate the effect of *Cinnamomum verum* on sporulation of coccidian oocysts by oocyst sporulation inhibition assay. The results revealed that body weight gain, feed conversion ratio and production efficiency factor declined considerably ($p < 0.05$) in positive control compared with negative control and concluded that 6 gram per Kg diet moderately reduced the coccidiosis and improved body weight and feed conversion ratio.

Sorheim *et al.* (2019) tested the antimicrobial activity of an aqueous extract of

Norwegian spruce bark, a waste product from the wood industry, in lambs. The results showed that there was a significant influence on coccidia development, as measured by faecal consistency, oocyst count and animal weight gain.

Crude methanol extracts of *Azadirachta indica*, *Vernonia amygdalina*, *Nicotiana tabacum*, *Moringa oleifera*, *Croton macrostachyus* and *Hagenia abyssinica* were screened *in vitro* for their potential anticoccidial effects against *E. tenella* (Wondimu *et al.*, 2021). The extracts were prepared in 100 mg/ml, 50 mg/ml and 25 mg/ml, whereas, amprolium 1.5 mg m⁻¹ and 2.5% K₂Cr₂O₇ served as positive and negative control, respectively. On phytochemical testing, extracts revealed the presence of saponins, tannins, alkaloids, steroids and glycosides and flavonoids, which may contribute for *in vitro* anticoccidial effects. The results revealed a dose and time dependent sporulation inhibition. At higher concentration (100 mg/ml) extract of *V. amygdalina*, *C. macrostachyus* and *A. indica* produced greater proportion of oocyst wall distortion.

In vivo anticoccidial activity of *Cinnamomum verum* bark (CNB) and *Rumex nervosus* leaves (RNL) in experimentally *E. tenella* infected broiler chickens was assessed by (Qaid *et al.*, 2022) in comparison with standard synthetic anticoccidial agent, salinomycin. The results revealed that RNL at 5 g and CNB at 6 g improved feed conversion efficiency and production efficiency index at 7 DPI compared to the infected groups indicating anti-coccidial activity.

2.3. Phytochemical studies:

Mathew and Parpia (1970) used P-dimensional paper chromatography to screen secondary metabolites of cashew kernel testa. Results revealed the presence of catechin and epicatechin, which were the major polyphenols.

Using hexane, ethyl acetate and methanol as solvents, Hoang *et al.* (2009) examined antioxidant activity of peanut skin extracts prepared from ordinary and high-oleic peanuts. The content of polyphenolic substances was low in hexane extracts, very high in methanolic extracts and intermediate in ethyl acetate extracts. Condensed tannins

prevailed in ethyl acetate and methanol extracts, while tocopherols were concentrated in hexane extracts, but only in moderate amounts. The resveratrol content was very low. The antioxidant activity of extracts was rather low in lard and in vegetable oils under conditions of the Schaal oven test, but the free radical-scavenging activity was very pronounced, especially in methanol extracts.

Shah *et al.* (2012) conducted phytochemical analyses of *Eucalyptus globulus* leaves by methanol, chloroform, petroleum ether and aqueous extraction. The analysis revealed carbohydrates, phytosterols and triterpinoids in petroleum ether extract, while carbohydrates, phytosterols and triterpinoids in chloroform extracts, phytosterols, phenolic compounds and tannins in methanol extract and triterpinoids, saponin and flavonoids in aqueous extracts.

Vinoth *et al.* (2012) used ethanol, methanol and acetone as solvents to screen phytochemicals from *Azadirachta indica* leaves. Reducing sugar, flavonoids, saponins and tannins were found in the ethanol extract, reducing sugar and glycosides were found in the acetone extract and reducing sugar, glycosides and terpenoids were found in the methanol extract.

The phytochemical content of *Eucalyptus globulus* leaf was analysed using ethyl acetate, methanol, hexane and water (Ishnava *et al.*, 2013). In hexane extract, steroids, cardiac glycosides, terpenoids, phenolic compounds and alkaloids were found, whereas, cardiac glycosides, terpenoids, phenolic compounds and steroids were found in ethyl acetate extract. More phytochemicals, such as tannins, saponin, cardiac glycosides, steroids, phenolic compounds and terpenoids, were found in the methanol extract.

The extracts obtained from *Anacardium occidentale* leaf were investigated for antioxidant and antimicrobial activities (Ajileye *et al.*, 2015). Bioassay-guided fractionation using DPPH autobiographic analysis of the EtOAc soluble fraction of the crude extract resulted in the isolation of agathisflavone and a mixture of quercetin 3-O-

rutinoside and quercetin 3-O-rhamnoside. The fractions of the extract showed comparatively better activities against Gram-negative than the Gram positive ones with the broadest spectrum of activities demonstrated for the ethyl acetate fraction. The isolated compounds from the plant possess essentially inhibitory rather than cidal activities against these organisms with the minimum bactericidal concentrations (MBCs) >2.0 mg/ml in all cases. This study concluded that the extracts and isolated compounds had strong antioxidant and moderate antibacterial activities and could be effective in the management of oxidative stress related diseases.

Meshkini (2016) screened for the polyphenol content and antioxidants of almond hulls. The phosphomolybdenum method demonstrated that, among several extraction methods, the acetone extract of almond hulls had a high amount of phenolic and flavonoid components and a high antioxidant activity. Almond hulls provided protection against the oxidative damage and membrane protein degradation produced by hydrogen peroxide in human erythrocytes. Finally, it was concluded that almond husk extract is a natural source of antioxidants and that its ingestion is beneficial to human health.

Using methanol, water, and ethanol as solvents, (Asimuddin *et al.*, 2017) conducted phytochemical screening of three medicinal plants (*Phyllanthus emblica*, *Terminalia chebula* and *Eucalyptus globules*) which revealed tannins, saponins, terpenoids, glycosides and alkaloids in some samples, whereas tannins, terpenoids and glycosides were found in others.

Cheenickal and Mendez (2017) investigated the phytochemical effects of aqueous, methanol and ethanol extracts of *Azadirachta indica* leaves that were both fresh and shade dried. Alkaloids, flavonoids, resins, bitter, saponin, tannin, cardiac glycosides, steroids, reducing sugars and volatile oils were found in ethanol extracts in fresh and shade dried leaves. In both fresh and shade dried leaves, methanol extracts produced positive results for alkaloids, cardiac glycosides and reducing sugars. Water extracts from

both fresh and shade dried leaves, on the other hand, generated exclusively cardiac glycosides.

Akinyemi and Oyelere (2019) analysed the *Cocos nucifera* endosperm (coconut meat), endocarp (coconut hard shell) and leaf extracts in the solvents ethanol, methanol and ethylacetate, as well as water, at concentrations of 1, 2, 3, 4, and 5% to know the type of organic chemical present in it. The results revealed that volatile oil was only found in the endocarp extract at 1.2% ethylacetate and 5% ethanol concentrations; phenol was only found in the plant's endocarp extract; alkaloid, flavonoids were only found in the plant's leaf extract; and tannin and saponin were found in all of the plant's organs.

Using the Elevated Plus Maze test (EPM), Open Feld Apparatus test (OFA) and light/dark exploration test Hugar (2019) assessed the anti-anxiety impact of ethanolic extract of endocarp of *Cocos nucifera* in animals at doses of 125, 250 and 500 mg/kg (LDT). The findings showed that phytoconstituents in plants contain flavonoids, phenolic compounds, alkaloids and tannins. They had efficacy against a variety of CNS diseases in rats.

Qureshi *et al.* (2019) used 70% ethanol, hexane, chloroform, ethyl acetate and n-butanol to determine the total polyphenolic and total flavonoid contents in the hulls of almond (*Prunus dulcis*) and water for evaluation of antioxidant, antidiabetic and antimicrobial activities in mass spectrometry. Extract also contained organic compounds (epi) catechin, chlor. The antioxidant activity of n-butanol extract was determined to be the highest, with an IC₅₀ value of 76.04 g/ml.

Adedayo *et al.* (2021) used oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power to investigate phytochemical content and antioxidant potentials of methanolic extracts of whole and dehulled Bambara groundnut (*Vigna subterranea*) seeds (FRAP). Whole BGN seeds had much higher flavanol and flavonol content, as well as significantly higher *in-vitro* antioxidant activity, than dehulled BGN

seeds, according to the findings.

2.4. Thin layer chromatography:

The phytochemical components, thin layer chromatographic profile and UV analysis of *Citrullus lanatus* leaf extracts were examined by Alebiosu and Yusuf (2015). The phytochemical investigation identified saponins, alkaloids, flavonoids, phenols, steroids and triterpenes. TLC was performed with the following solvent systems: hexane (100%), hexane: ethylacetate (9: 1; 8: 2, 7: 3), chloroform: methanol (30: 1, 15: 1), chloroform: ethylacetate: methanol: water (15: 8: 4: 1), n-butanol: acetic acid: water (15: 8: 4: 1), chloroform: ethylacetate: (4: 1: 5). TLC analysis revealed homogenous spots with varied R_f values, showing that the *C. lanatus* leaf contains secondary metabolites and that a mobile phase that is suited for each fraction has been created.

Gade *et al.* (2016) used a methanol solvent to study the phytochemical screening and TLC of *Aegle marmelos* leaves against fungal infections. In the methanolic extract, phytochemical screening revealed the presence of alkaloids, tannins, flavonoids, saponin and phenolic chemicals. The solvent system used for TLC was Petroleum ether: Ethyl acetate (2:1), which yielded the most compounds. The R_F values for the various chemicals were 0.04, 0.07, 0.14, 0.18, 0.25, 0.35, 0.45, 0.61, 0.70 and 0.84, respectively.

Vani *et al.* (2016) carried out thin layer chromatography to characterize *Azadirachta indica* leaf extracts utilising ethanol, methanol, benzene, ethyl acetate and toluene as solvents for extraction. Thin layer chromatography revealed the presence of phytochemicals such as nimbin (R_f 0.09), which appears blue, ascorbic acid (R_f 0.74), amino acid (R_f 0.62), which appears pink, and azadirachtin (R_f 0.70), which appears light green.

Galib *et al.* (2017) carried out phytochemical investigations and thin layer chromatography (TLC) on *Acacia etabica* leaves utilising Dichloromethane (DCM), water and ethanol as extraction solvents. The solvent system used in TLC was ethyl acetate: toluene: formic acid (4: 4: 1). TLC of ethanolic extract showed six spots of red,

yellow, and light blue with Rf values 0.90, 0.85, 0.80, 0.79, 0.74 and 0.7; DCM extract showed six spots of red and pink with Rf values 0.8, 0.69, 0.6, 0.51, 0.47 and 0.43; and TLC of water extract showed one spot of light pink with Rf values 0.69.

Rashed *et al.* (2019) investigated *Moringa oleifera* extracts in methanol, ethanol, ethanol acetate and water for phytochemical screening and TLC. Chloroform: methanol: ethanol (1: 1: 1), (2: 2: 0.5) and chloroform: glacial acetic acid: methanol was utilised as solvent solutions for TLC (4: 5: 1). After phytochemical screening of four extracts, alkaloids, flavonoids, tannins and phenols were detected in all solvent extracts. Methanol and ethyl acetate extracts revealed four bands with Rf less than one when compared to ethanol and water, indicating that methanol and ethyl acetate extracts have more bioactive molecules.

CHAPTER-III

MATERIALS AND METHODS

The present work is carried out to study the *in vitro* anticoccidial activity of the nut by-products namely nut peel of cashew (*Anacardium occidentale*), endocarp of green coconut (*Cocos nucifera*), hulls of almond (*Terminalia catappa*) and partially developed groundnut kernel (*Arachis hypogea*) on oocysts of *Eimeria* species of poultry.

3.1. Materials

3.1.1. Glassware

The glassware used in this study was obtained from Borosil and were thoroughly washed and sterilized as per the standard procedure. Other laboratory wares such as tubes and micro tips were procured from Tarsons. Trueline 24 well - cell culture plates (TR5002 24 well) were used for sporulation inhibition assay.

3.1.2. Chemicals

The chemicals, Dragendroff's reagent, lead acetate, 5% Ferric chloride, chloroform, concentrated H₂SO₄, glacial acetic acid, Benedict's reagent, 0.2% Ninhydrin, potassium dichromate, sodium chloride, potassium chloride, disodium hydrogen ortho phosphate, potassium dihydrogen orthophosphate, sodium acetate, HCl, dimethyl sulfoxide (DMSO), Salinomycin (12% pure standard reference), Cosmo Dot- EP (DOT + Ethopabate feed additive) and 10% formalin used in the present study were of analytical or guaranteed reagent grade.

3.1.3. Instrumentation

The instruments GR 200 Weighing balancer, pH meter 720 inoLab (M/S Merck, Bengaluru), YSI-402 Yorco Autoclave vertical (National Scientific, Hyderabad), Milli-Q water plant (Millipore, USA), Orbital Shaker (Thermo-scientific, USA), Serological hot water bath (Yorco), Micropipette (M/S Eppendorf, USA), Ocular Micrometer (M/S National Scientific, Hyderabad), Stage Micrometer (M/S National Scientific, Hyderabad), Hot air oven (M/S Deccan Techno Corporation, Hyderabad), Yorco High

Precision water bath (M/S National Scientific), Fluorescent microscope (Olympus BX 40), Magnetic stirrer (GENEI, Bangalore), McMaster slide (Annapurna scientifics, Hyderabad) were used during the study.

3.1.4. Plant collection

Plants selection was based on the literature survey on the traditional uses of the plants in India and other parts of the world. Those plants with claimed anthelmintic activity but not evaluated for the activity against poultry coccidia were selected. Nut peel of cashew (*Anacardium occidentale*) (Plate 1) was collected from its natural habitat Srikakulam while the endocarp of green coconut (*Cocos nucifera*) (Plate 3), hulls of almond (*Terminalia catappa*) (Plate 2), and partially developed groundnut (*Arachis hypogea*) (Plate 4) were purchased from local market of Andhra Pradesh.

Table.1. Parts of plants evaluated for anticoccidial activity

S. No	Plant species	Part used	English name	Vernacular name
1.	<i>Anacardium Occidentale</i>	Nut peels	Cashew	Zeedipappu
2.	<i>Terminalia catappa</i>	Hulls	Almond	Badam
3.	<i>Cocos nucifera</i>	Endocarp	Coconut	Kobbari
4.	<i>Arachis hypogea</i>	Partially developed kernel	Groundnut	Verushanaga

3.2. Methods

3.2.1. Sterilization of glassware

The glassware used in this experiment was steeped in labolene overnight and then rinsed several times with tap water before being submerged in acid water (H₂SO₄) overnight. The glassware was then rinsed multiple times under running water and autoclaved at 121°C for 15 minutes at 15 lbs of pressure before being sterilised in a hot air oven at 100°C for an hour.

3.2.2. Preparation of 2.5% potassium dichromate (2.5% K₂Cr₂O₇):

Potassium dichromate (2.5%) solution was prepared by dissolving 2.5 g of potassium dichromate in 100 mL of distilled water and was stored for further use in sporulation inhibition assay.

3.2.3. Preparation of phosphate buffer saline (pH 7.2):

S.NO.	Ingredients	Quantity
1.	Sodium chloride (NaCl)	8.0 g
2.	Potassium chloride (KCl)	0.2 g
3.	Disodium hydrogen ortho phosphate (Na ₂ HPO ₄)	1.133g
4.	Potassium dihydrogen orthophosphate (KH ₂ PO ₄)	0.2 g
5.	Distilled water	1000 ml

Sterilised by autoclaving at 121 °C for 15 minutes.

3.2.4. Preparation of floatation fluid:

Saturated sodium chloride solution was prepared (specific gravity 36 gm/100 ml) as described by Fatemi *et al.* (2015).

3.2.5. Preparation of plant extracts

The nut peel of cashew (*Anacardium occidentale*), endocarp of green coconut (*Cocos nucifera*), hulls of almond (*Terminalia catappa*) and partially developed groundnut (*Arachis hypogea*) were thoroughly washed thrice with double distilled water to remove the dirt, soil particles from the surface, sliced into pieces, shade dried without exposure to sun rays for several weeks and crushed into coarse powder using a kitchen grinder and were stored in air tight container.

3.2.6. Preparation of methanolic extracts

Four conical flasks with a capacity of 500 ml were used. Twenty grams of the dried and powdered plant material was mixed in a conical flask containing 200 ml of 100% methanol. The conical flask was closed air tight with non absorbent cotton and aluminium foil. The material was subjected to constant stirring at 100 RPM in orbital shaker for

overnight at room temperature. The remnant was separated by filtration through Whatman filter paper No.1 (Plate 5). The solvent in the filtrate was then evaporated in a water bath at 50⁰C and on magnetic stirrer until a consistent solid mass was obtained. Finally, this dried methanolic extracts were re-suspended in dimethyl sulfoxide (DMSO) for obtaining a final concentration of 1000 mg/ml or 500 mg/ml of stock solution (plate 6) and were then stored in refrigerator at 4⁰C until further use. Extraction yield was calculated using the following formula:

$$\text{Extraction yield (\% w/w)} = (w1 \times 100) / w2$$

w1 = weight of the extract obtained after evaporation of solvent.

w2 = weight of the plant powder.

3.3. Thin layer chromatography

Thin layer chromatography (TLC) was employed to determine the number of chemical components found in the plant extracts. The different solvent systems of different polarities were prepared and TLC studies were carried out to select the solvent system showing capable of better resolution.

Solvent phase:

The different solvent systems used were

1) methanol: toluene: acetone: water (30:30:10:30)

2) ethyl acetate: methanol: chloroform (20:20:60)

3) ethyl acetate: methanol: water (50:30:20)

Methodology:

Thin layer chromatography was performed on each of the four extracts on analytical plates over silica gel (TLC Silica gel 60 F254; Merck India). The TLC plates were cut to the size of a glass slide and activated in a hot air oven at 90⁰C for 5 minutes. For each extract, three different solvent systems viz., methanol: toluene: acetone: water (30: 30: 10: 30); ethyl acetate: methanol: chloroform (20: 20: 60), ethyl acetate: methanol: water (50: 30: 20) were used as mobile phase. Each TLC plate was treated with ten

microliters of cashew shell extract before placing in the developing chamber. The TLC plates were air dried once the extract movement in the solvent solution ceased. In a TLC plate reader, the UV absorbance was measured at 254 and 365 nm. Almond hulls, coconut endocarp and groundnut kernel extracts were also subjected to the same procedure.

3.4. Phytochemical screening of extracts

Four methanolic extracts were screened for the presence of phytochemicals such as reducing sugars, proteins, lipids and secondary metabolites in particular alkaloids, flavonoids, tannins, saponins, polyphenols, terpenoid, cardiac glycosides and steroids qualitatively. These procedures were developed based on visual observation of colour change or precipitate development after the addition of specified chemicals. Phytochemical tests were conducted in 2 ml centrifuge tubes.

Test for alkaloids:

Methanolic extract (0.5 ml) was mixed with 2-3 drops of Dragendroff's reagent. Alkaloids were found in the form of an orange-red precipitate/turbidity.

Test for flavonoids:

A few drops of lead acetate solution were added to 0.5 ml of methanolic extract. The presence of flavonoids was noticed by the formation of yellow precipitate. Flavonones are present when the colour changes from orange to crimson.

Test for saponins:

A mixture of 0.5 ml extract and 1.0 ml distilled water was vigorously shaken. Saponins were detected by continuous foam that lasted for at least 15 minutes.

Test for tannins:

The test was done by adding 2-3 drops of 5% ferric chloride solution to two tubes holding 0.5 ml extract each, and the mixture was agitated. The presence of hydrolysable tannins was indicated by the production of dark blue precipitate, while the presence of condensed tannins was shown by the presence of green precipitate.

Test for triterpenoids:

To test for triterpenoids, 0.5 ml of methanolic extract was mixed with 1.0 ml of chloroform. Concentrated H₂SO₄ (0.5 ml) was carefully added to form a layer. Appearance of reddish brown colour at the interface was indicative of presence of terpenoids.

Test for cardiac glycosides:

A drop of ferric chloride solution was added to 0.5 ml of methanolic extract in 1.0 mL of glacial acetic acid to which 0.5 ml concentrated H₂SO₄ was subsequently carefully added to form a layer. The deoxysugars characteristic of cardenolides was shown by a brown ring at the interface. A violet ring might appear beneath the brown ring, while a greenish ring formed progressively over the thin acetic acid layer.

Test for carbohydrates:

The test was performed by combining 0.5 ml methanolic extract with a few drops of Benedict's reagent (an alkaline solution containing cupric citrate complex) and kept at 40°C in a water bath. The presence of reducing sugars was shown by the formation of a reddish brown precipitate at the tube's bottom.

Test for protein:

Two drops of Ninhydrin 0.2% solution were added to 0.5 ml of extract and kept at 40°C in a water bath. The presence of amino acids and protein was indicated by the violet colour.

3.5. Preparation of positive control

Salinomycin (12% pure standard reference) obtained from PVS Laboratories Ltd., Vijayawada, and Cosmo Dot- EP from Department of Veterinary Nutrition, NTR College of Veterinary Science, Gannavaram, Krishna district, Andhra Pradesh was initially dissolved in DMSO and diluted in PBS at the desired concentrations to use in different assays. Similarly plant extracts were also diluted in PBS to desired concentrations.

3.6. Sporulation inhibition assay

3.6.1. Collection of faecal samples:

Faecal samples were collected from birds naturally infected with coccidiosis in poultry farms in and around Krishna district to achieve high quantum of oocysts. Immediately after sample collection, samples were brought to the Department of Veterinary Parasitology, NTR College of Veterinary Science, Gannavaram, Krishna district.

3.6.2. Oocyst separation from faeces:

Recovery and preparation of unsporulated oocysts for bioassay was done as per the method described by (Ryley *et al.*, 1976) with minor modifications.

1. Water was slowly added to the faecal pellets, homogenised in a mortar and pestle until a relatively liquid suspension was obtained and organic debris was removed by filtration through a sieve (3 mm).

2. The suspension that passed through the sieve was collected and washed through 8 inch diameter 40 and 100 mesh sieves (390 and 150 μm pore size).

3. Thereafter, the suspension was collected into 15 ml centrifuge tubes and centrifuged for 5 minutes at 3000 RPM.

4. After decanting the supernatant, tubes were agitated to loosen the sediment, filled with saturated sodium chloride solution and re-centrifuged for 5 min at 3000 RPM.

5. The floating oocysts were removed by pouring the supernatant through a sieve into a conical glass centrifuge tube.

6. Centrifuge tube was filled with distilled water and centrifuged at 3000 RPM for 5 min.

7. Supernatant was decanted and sediment containing the oocysts was re-suspended in

PBS and repeated the procedure for three times. Finally sediment was suspended in PBS to form required quantity of oocysts.

3.6.3. Oocyst count by Mc Master method

Oocyst per gram (OPG) of faeces was calculated by Mc Master method as suggested by (Molan *et al.*, 2009). Three grams of the faecal sample was mixed thoroughly with 42 ml of saturated sodium chloride in a beaker and the chambers of the McMaster slide were filled with the suspension and allowed to stand undisturbed for 2-3 minutes, so that oocysts came into the same focus level against the ruled area. The McMaster slide was examined microscopically under 10x magnification and the OPG was calculated by utilizing the standard formula (Soulsby, 1982). In addition, one drop of this suspension was drawn by means of graduated pipette and the total number of unsporulated oocysts was counted under microscope to calculate the number of oocysts to be used in each well. Average of six such observations were taken and the number of unsporulated oocysts in a drop and total dose (2500 oocysts/0.5 ml) was calculated.

3.6.4. Microscopy of unsporulated oocysts for identification of different species

Final suspension of unsporulated oocyst was subjected to morphometric analysis by microscopic examination and species differentiation is carried out by their shape and size (Reid and Long, 1979). Morphometric analysis of unsporulated oocysts revealed the presence of *E. tenella*, *E. necatrix*, *E. maxima*, *E. brunetti* and *E. acervulina* in the laboratory stock solution.

3.6.5. Procedure of sporulation inhibition assay

1. The test was performed in 35 mm diameter glass Petri dish (Borosil) and Trueline 24 well-cell culture plate (TR5002 24 well) as per the procedure of Zaman *et al.* (2015) for four extracts.
2. The oocyst suspension was mixed thoroughly to get a uniform suspension of oocysts.
3. Then, the unsporulated oocysts were mixed in 2.5% potassium dichromate solution

(aqueous) in 1:5 ratios.

4. Oocysts (2500 oocysts/0.5 ml) were exposed thrice in three replicates to each of the three fold dilution of plant extracts (300, 100, 30, 10, 3 mg/ml) in separate culture plates (Plate 7 to 10).

5. Besides, a positive (salinomycin (60 ppm), CosmoDot- EP at concentrations of 0.5 mg/ml and 10% formalin), and negative controls (2.5% $K_2Cr_2O_7$) were included in the assay.

6. Finally the plates were incubated at 25–29°C with 80% relative humidity for 48 hours.

7. After 48 hours; the oocysts exposed to plant extracts, salinomycin, CosmoDot- EP, 10% formalin and 2.5% $K_2Cr_2O_7$ were washed twice in tap water by centrifuged at 3000 RPM for 5 minutes and stored at 4°C until being counted with light microscope.

8. The number of sporulated and unsporulated oocysts was counted and the percent sporulation was estimated by counting the number of sporulated oocysts in a total of 100 oocysts. In addition, the numbers of abnormal (in terms of shape and size) oocysts were also noticed (Molan *et al.*, 2009).

9. Sporulation inhibition (SI) was determined in percentage according to the following equation as described by (You, 2014).

$$SI \% = \frac{\text{Sporulation of control} - \text{sporulation of treated}}{\text{sporulation of control}} \times 100$$

3.7. Statistical Analysis

Data from assays was transformed by probit analysis against the logarithm of extract concentration. Data were calculated for estimating ED_{50} by using logtime probit model using the LdP LineR software (LdP Line, Copy right 2000 by Ehab Mostofa Bakr, Cairo, Egypt). Comparison of mean percentages of sporulation inhibition at different concentrations was performed by one-way ANOVA using SPSS version 25.0. The post

hoc statistical significance test employed was least square difference (LSD), the difference between the means was considered significant at $P < 0.01$.

CHAPTER – IV

RESULTS

The present study was conducted to evaluate the anticoccidial efficacy of nut by-products namely nut peel of cashew (*Anacardium occidentale*), endocarp of green coconut (*Cocos nucifera*), hulls of almond (*Terminalia catappa*) and partially developed groundnut kernel (*Arachis hypogea*) on *Eimeria* species of poultry by using sporulation inhibition bio assay and to determine the secondary metabolites of these extracts.

On microscopic examination of faecal samples collected from 492 birds from poultry farms and slaughter houses in and around Krishna district, Andhra Pradesh; 385 were found positive for oocysts of *Eimeria* species with a prevalence of 78.25 per cent. Further morphometric analysis of unsporulated oocysts revealed the presence of *E. maxima* (40%), *E. tenella* (20%), *E. necatrix* (20%), *E. brunetti* (5%) and *E. acervulina* (5%) oocysts (Plate 11).

4.1. Extraction and screening of plant materials

4.1.1. Extraction

The methanolic extracts of selected plant materials, displayed difference in percent yield among them (Table 1 and Fig. 1). The highest percent yield was obtained for the hulls of *Terminalia catappa* (26.32%) and the lowest percent recovery (2.3%) was recorded for partially developed *Arachis hypogea*.

Table 2. Physical characteristics and percentage yield of the different plants

S.No	Botanical name	Parts used	Extract colour	Dry powder weight (gm)	Odour	% yield (w/w)
1.	<i>A. occidentale</i>	Peel	Dark brown	20	Peculiar	8.45
2.	<i>T. catappa</i>	Hulls	Brown	20	Peculiar	26.32

3.	<i>C. nucifera</i>	Endocarp	Yellow	20	Peculiar	14.42
4.	<i>A. hypogea</i>	Partially developed	Golden Yellow	20	Aromatic	2.3

w/w: weight by weight

4.1.2. Phytochemical screening

The phytochemical screening of the methanolic extracts of *A. occidentale* nut peel, *T. catappa* hulls, *C. nucifera* endocarp, and *A. hypogea* partially developed nuts revealed the presence of compounds known to have various pharmacologic activities and therapeutic uses: flavonoids (Plate 12), tannins (Plate 13), saponins (Plate 14), carbohydrates (Plate 15), proteins (Plate 16) and triterpenoids (plate 17). The principal chemical constituents identified from the medicinal plants are indicated in Table 2. All the extracts tested had major secondary metabolites like flavonoids, tannins, triterpenoids and saponins. Alkaloids were found to be present exclusively in the peel of *A. occidentale* and endocarp of *C. nucifera*. Other metabolites such as cardiac glycosides were not found in any plant extract.

Table 3. Phytochemical screening of plant extracts

S.No.	Chemical constituents	Tests	<i>Anacardium occidentale</i>	<i>Terminalia catappa</i>	<i>Cocos nucifera</i>	<i>Arachis hypogea</i>
1	Tannins	Ferric chloride test	+++	++	+++	+++
2	Saponins	Foam test	+++	+++	++	++
3	Alkaloids	Dragendroff test	++	-	++	-

4	Flavonoids	Lead acetate test	+++	+++	++	+
5	Cardiac glycosides	Keller-kilani test	-	-	-	-
6	Triterpenoids	Salkowski test	++	+	++	++
7	Amino acids	Ninhydrin test	-	-	-	+
8	Carbohydrate	Benedicts test	+++	+++	+++	+

- :Negative; +: Weak coloration; ++: Moderate coloration; +++: Strong coloration

4.1.3. Thin layer chromatography

A variety of solvent systems namely methanol: toluene: acetone: water (30: 30: 10: 30); ethyl acetate: chloroform: methanol (20: 60: 20); and ethyl acetate: methanol: water (50: 30: 20) were tried to achieve good resolution. Finally, the solvents ethyl acetate: chloroform: methanol (20: 60: 20); and ethyl acetate: methanol: water (50: 30: 20) were considered. The presence of various flavonoids and phenolic compounds were observed under 365 nm UV light, (dark fluorescent dots) (Plate 20). Whereas, polyphenols were observed under UV light at 254 nm in the extracts (Plate 18 and 19) and these chemicals are noticed as arc shaped, blue in colour and make up the majority of the extract, are most likely tannins, flavonoid compounds and are high in *A. occidentale* extract compared with the other extracts.

4.2. Anticoccidial effect of plant extracts on *Eimeria*: *In vitro* experiments

4.2.1. Sporulation inhibition assay (SIA)

Sporulation inhibition (SI) was determined in percentage.

4.2.1.1. SIA with *A. occidentale* peel extract

The methanolic extract of *A. occidentale* nut peel at 300 mg/ml concentrations revealed 88.58 ± 0.39 per cent inhibition of sporulation. At the same time, the

concentrations of 100, and 30 mg/ml inhibited the sporulation at a rate of 83.07 ± 2.76 and 78.34 ± 2.58 per cent, respectively. Similarly, extracts at the concentration of 10 and 3mg/ml, inhibited the sporulation of oocysts at a rate of 78.34 ± 4.84 and 75.19 ± 4.47 per cent, respectively. The inhibition of sporulation in the reference drugs, salinomycin (60 ppm), ethopabate (0.5 mg/ml) and formalin (10%) (positive control) was 92.91 ± 1.80 , 84.64 ± 2.73 and 98.03 ± 1.04 per cent, respectively. The mean inhibition of sporulation for negative control was 15.33 ± 0.88 per cent.

4.2.1.2. SIA with *T. catappa* hull extract

The methanolic extract of *T. catappa* hulls at 300 mg/ml concentration revealed 79.24 ± 0.83 per cent inhibition of sporulation. However, the concentrations of 100, and 30 mg/ml inhibited the sporulation process at a rate of 77.99 ± 0.42 and 74.68 ± 2.91 per cent. The per cent of sporulation inhibition was 71.77 ± 2.31 and 71.36 ± 2.16 respectively, at 10 and 3 mg/ml concentrations. The inhibitory effect of salinomycin (60 ppm), ethopabate (0.5mg/mL) and formalin (10%) was 90.03 ± 1.90 , 83.81 ± 1.44 and 96.68 ± 1.81 per cent, respectively. The mean inhibition of sporulation for negative control was 19.67 ± 0.88 per cent.

4.2.1.3. SIA with *C. nucifera* endocarp extract

The methanolic extract of *C. nucifera* at 300 mg/ml concentration revealed 8.07 ± 2.34 per cent inhibition of sporulation. Whereas, the concentrations of 100, and 30 mg/ml inhibited the sporulation process at a rate of 5.38 ± 2.40 and 3.84 ± 3.00 per cent, respectively. The concentrations of 10, and 3 mg/ml, showed 1.15 ± 3.35 and 1.15 ± 2.78 per cent sporulation inhibition, respectively. Whereas, the positive controls such as salinomycin (60 ppm), ethopabate (0.5mg/ml) and formalin (10%) induced 89.99 ± 1.54 , 86.15 ± 1.99 and 97.31 ± 1.02 per cent sporulation inhibition, respectively. The mean inhibition of sporulation for negative control was 13.33 ± 1.77 per cent.

4.2.1.4. SIA with *A. hypogea* (partially developed kernel) extract

The methanolic extract of *A. hypogea* kernels at 300 mg/ml concentration revealed 1.43 ± 0.75 per cent inhibition of sporulation. The concentrations of 100 and 30 mg/ml induced the inhibition of 1.05 ± 1.64 and 0.68 ± 1.72 per cent, whereas at the concentrations of 10 and 3 mg/ml, the extracts manifested 0.07 ± 1.36 and -0.07 ± 1.36 per cent inhibition, respectively. The rate of sporulation inhibition by salinomycin (60 ppm), ethopabate (0.5mg/ml) and 10% formalin was 93.23 ± 1.30 , 85.32 ± 1.95 and 94.36 ± 1.30 per cent, respectively. The mean inhibition of sporulation for negative control was 11.33 ± 2.03 per cent.

4.2.1.5. Comparative analysis of anticoccidial effect of different extracts

The methanolic extracts of *A. occidentale* nut peel and *T. catappa* hulls at the end of 48 h of exposure showed significant ($P < 0.01$) inhibitory effect on sporulation of *Eimeria* oocysts in comparison with negative control, with reference drugs being more effective than the plant extracts. The inhibition of sporulation was not dose dependent for *A. occidentale* peel and *T. catappa* hulls extracts. At highest concentration (300 mg/ml) the rate of sporulation inhibition by *C. nucifera* endocarp and partially developed *A. hypogea* kernels was 8.07 ± 2.34 per cent and 1.43 ± 0.75 per cent, respectively, which was imperceptible in comparison with reference drugs.

The methanolic extracts of *A. occidentale* nut peel and *T. catappa* hulls exhibited good sporulation inhibition against oocysts of *Eimeria* although there was no significant variation in mean inhibition at different dose levels for each type of extract (Table 3). Independent comparisons revealed that at the concentration of 100 mg/ml or higher, the percentage inhibition of *A. occidentale* peel was significantly higher ($P < 0.05$) than that of *T. catappa* hulls. Methanolic extracts of *A. occidentale* nut peel induced nearly 90% per cent sporulation inhibition at highest concentration of 300 mg/ml. The effective doses required to induce 50% (ED_{50}) inhibition of sporulation was calculated by probit analysis

(Table 5). Extracts of *A. occidentale* nut peel induced 50% inhibition at lower concentration (1.099 µg/ml) compared to *T. catappa* extract (7.627 µg/ml).

4.3. Morphological alterations in *Eimeria* oocysts after extract exposure

All four extracts induced alterations in the normal morphology and shape of oocysts of *Eimeria* species at all concentrations. There was no change in morphology of oocyst in control groups except in 10% formalin. Abnormal oocysts from incubations containing herbal extract showed changes in oocyst size and wall morphology such as boat shaped oocysts (Plate 21), pitted oocyst wall (Plate 21), rupture of oocyst wall (Plate 22), compressed oocyst wall (Plate 23), wrinkled oocysts (Plate 24), leakage of oocyst mass (Plate 24) and abnormality in size and shape of sporocysts (Plate 23) were also observed.

Plate 1: Peel of cashew nut (*A. occidentale*)



Plate2: Hulls of almond (*T. catappa*)



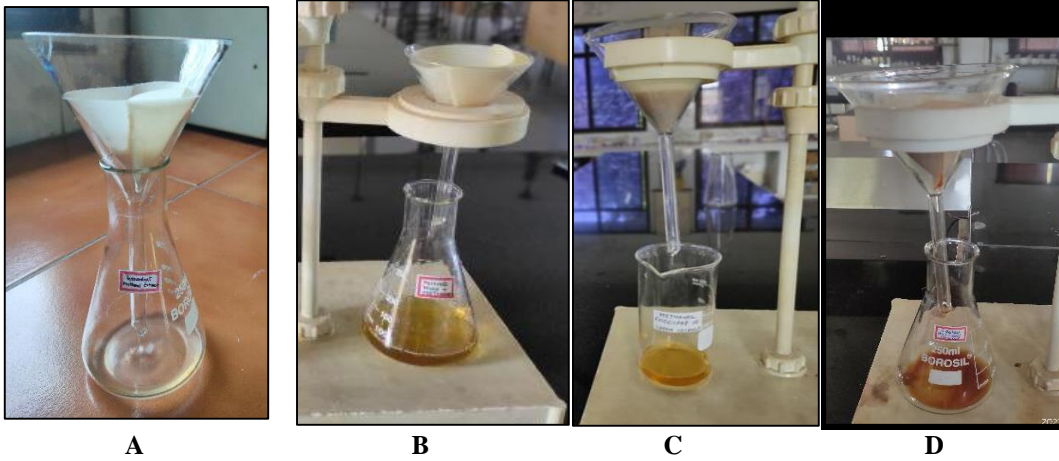
Plate 3: Endocarp of green coconut (*C. nucifera*)



Plate 4: Partially developed groundnut (*A. hypogea*) kernels



Plate 5: Filtration of all the extracts through Whatmann's filter paper no.1



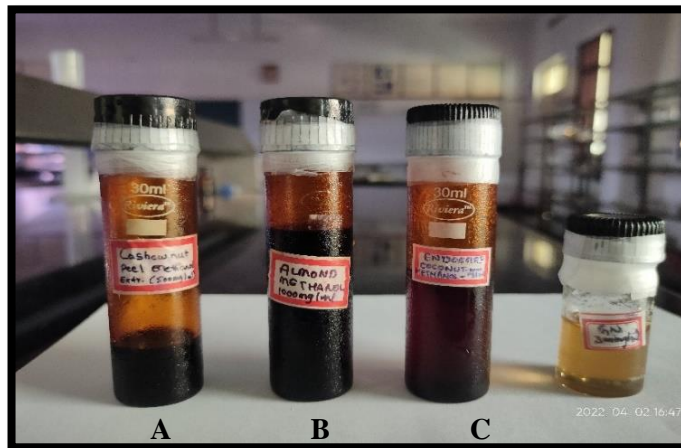
A

B

C

D

Plate 6: Preparation of stock solutions



A

B

C

A - *A. occidentale*
 B - *T. catappa*
 C - *C. nucifera*
 D - *A. hypogea*

Plate 7: SIA with *A. occidentale* extract



Plate 8: SIA with *T. catappa* extract

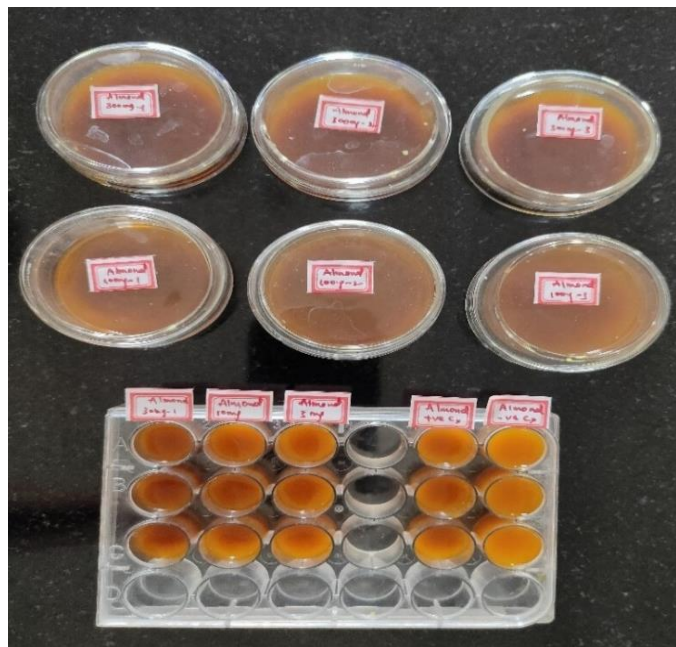


Plate 9: SIA with *C. nucifera* extract

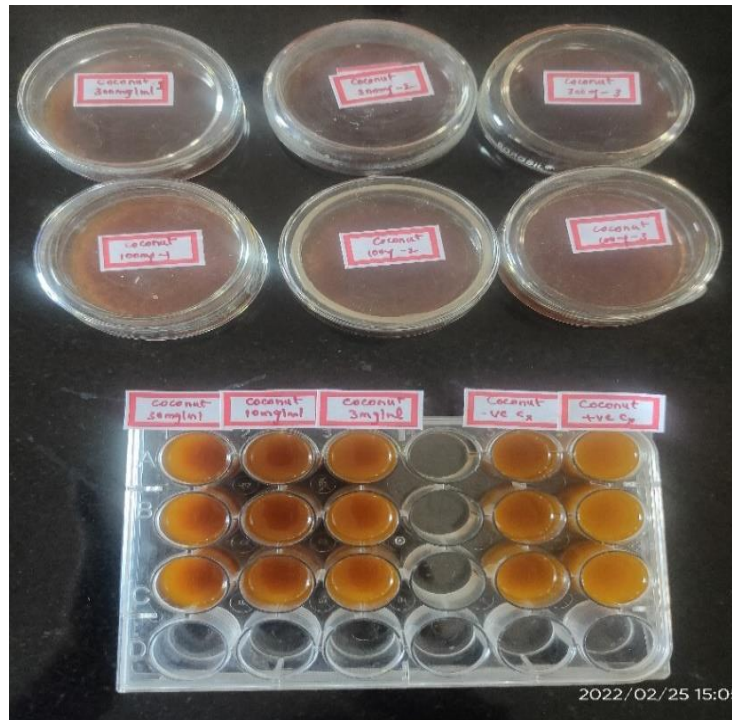


Plate 10: SIA with *A. hypogea* extract

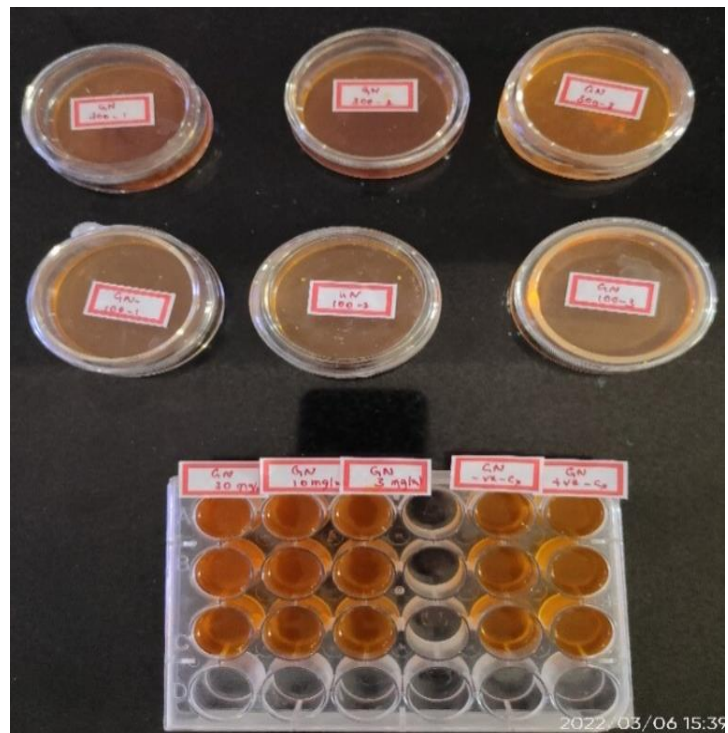
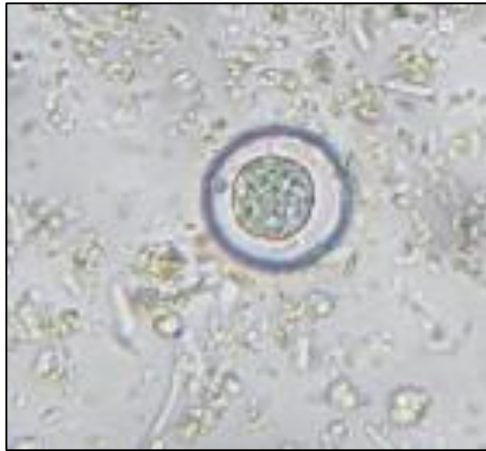
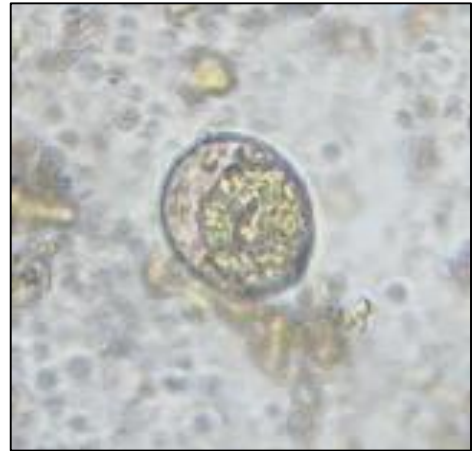
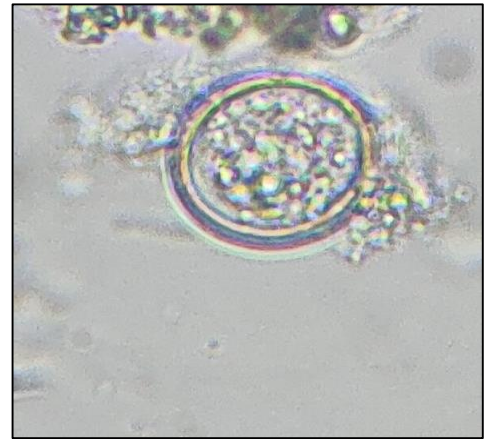


Plate 11: Species specific identification of *Eimeria* oocysts***E. acervulina* (27x25µm)*****E. brunetti* (26.5x22.5 µm)*****E. maxima* (30x25 µm)*****E. necatrix* (20x17.5 µm)*****E. tenella* (20x19 µm)**

Phytochemical analysis

Plate 12: Lead acetate test: Yellow precipitate indicates the presence of flavonoids

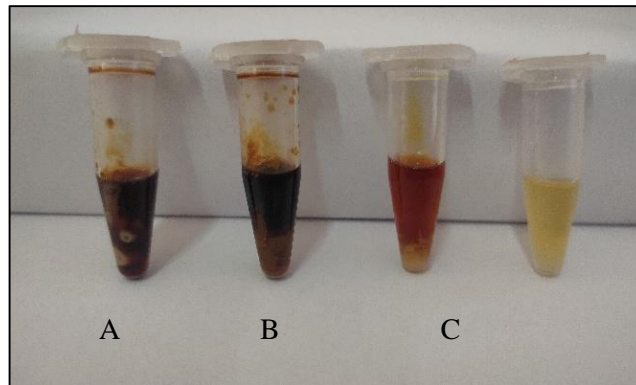


Plate 13: Ferric chloride test: Dark blue colour indicates the presence of tannins

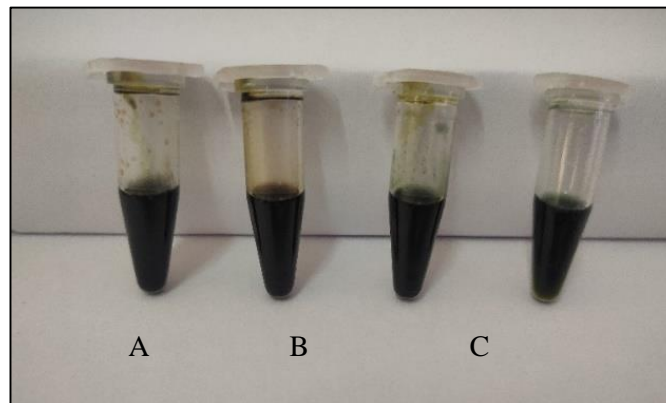
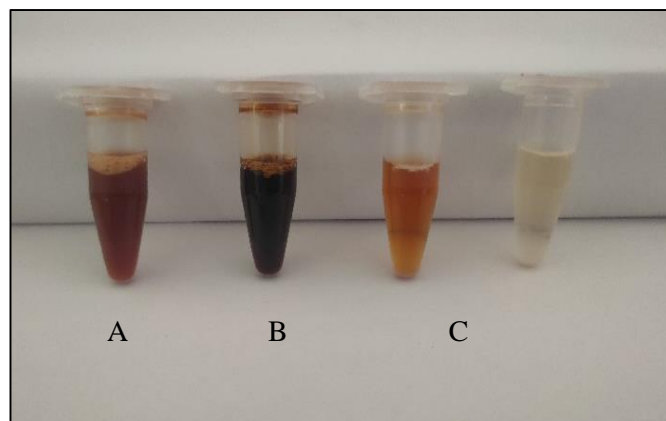


Plate 14: Foam test: Froth indicates the presence of saponins



A - *A. occidentale*
B - *T. catappa*
C - *C. nucifera*
D - *A. hypogea*

Plate 15: Benedict's test: Dark brown precipitate indicates the presence of Carbohydrates

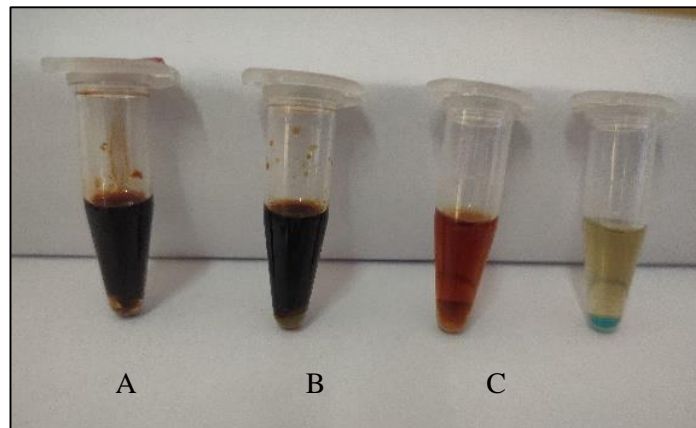


Plate 16: Ninhydrin test: Violet colour indicates the presence of proteins

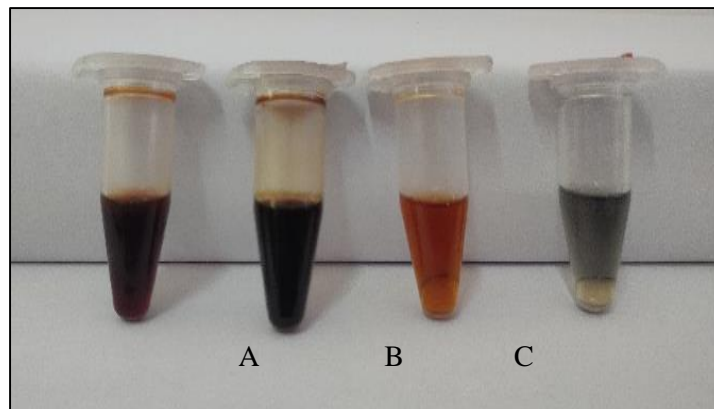
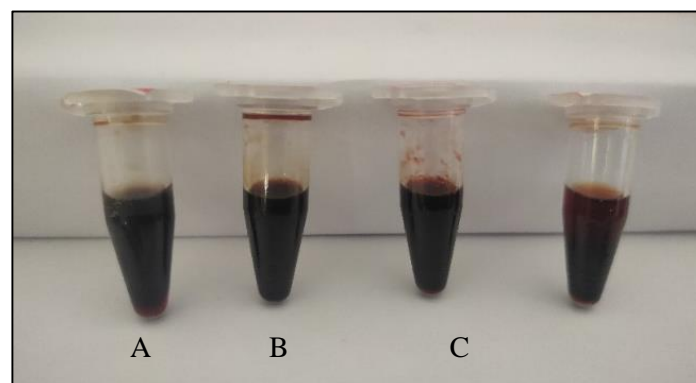


Plate 17: Terpenoids test: Reddish brown colour at the interface indicates the presence of terpenoids.



- A - *A. occidentale*
- B - *T. catappa*
- C - *C. nucifera*
- D - *A. hypogea*

Plate 18: TLC of *A. occidentale*, *T. catappa*, *C. nucifera* and *A. hypogea* extracts
Solvent system: ethyl acetate: chloroform: methanol (20:60:20) UV-254 nm

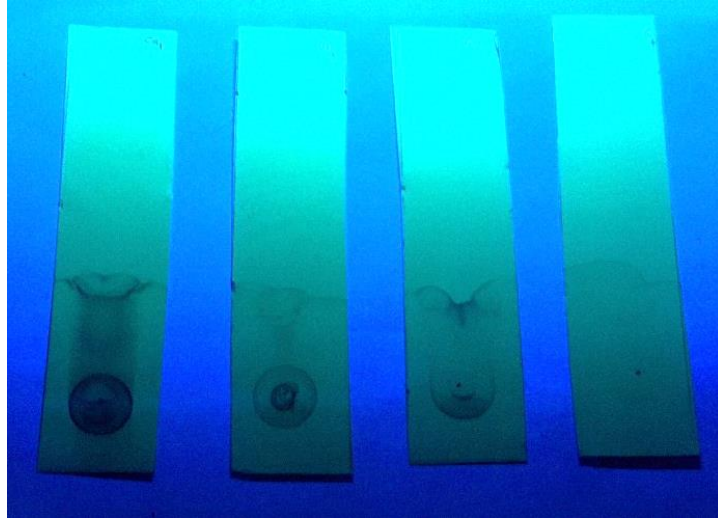


Plate 19: TLC of *A. occidentale*, *T. catappa*, *C. nucifera* and *A. hypogea* extracts
Solvent system: ethyl acetate: methanol: water (50:30:20) UV-254 nm

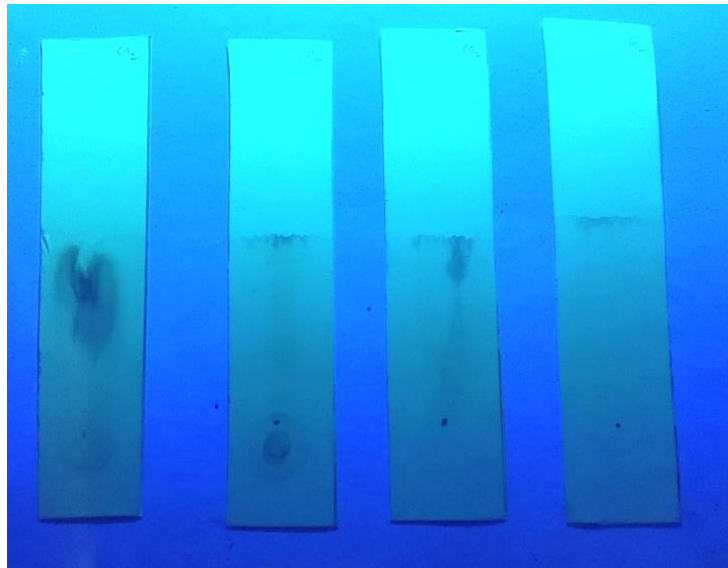


Plate 20: TLC of *A. occidentale*, *T. catappa*, *C. nucifera* and *A. hypogea* extracts
Solvent system: ethyl acetate: methanol: water (50:30:20) UV-365 nm

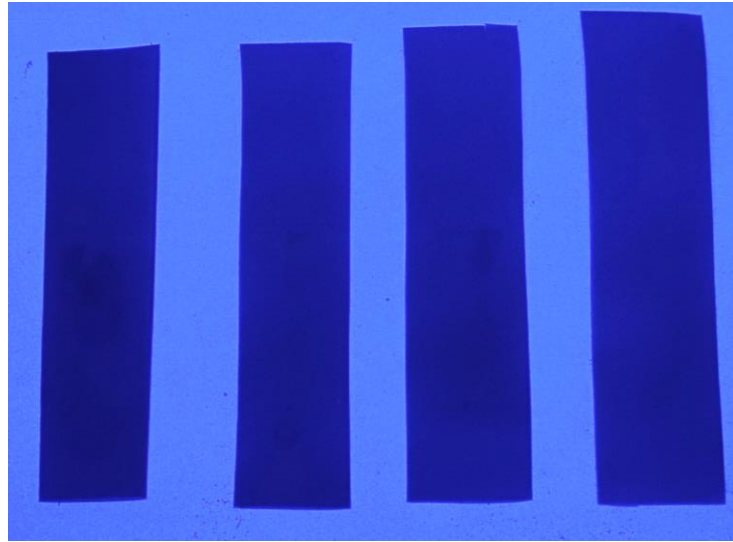


Plate 21: Morphological alterations of unsporulated oocysts treated with
A. occidentale extract

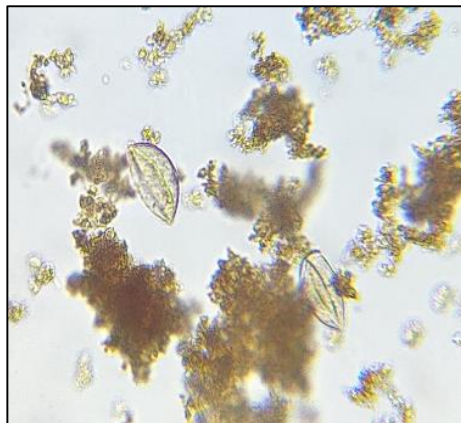
Damaged oocyst wall



Compressed oocyst wall



Boat shaped oocysts



Pit on oocysts

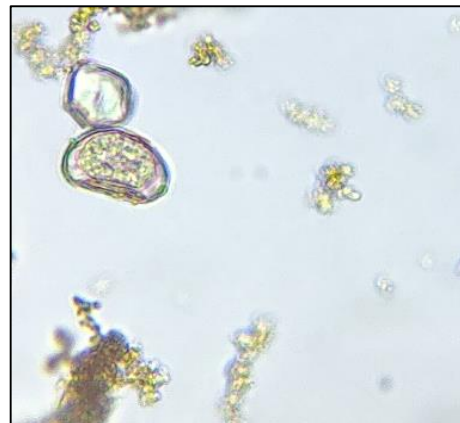


Plate 22: Morphological alterations of unsporulated oocysts treated with *T. catappa* extract

Rupture of oocyst mass

Damaged oocysts

Variation in shape of oocyst wall

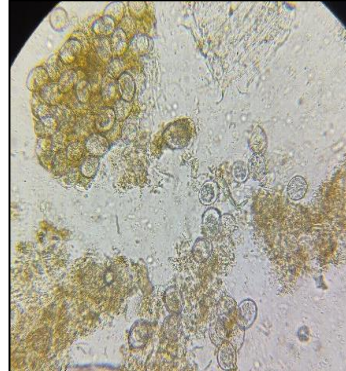


Plate 23: Morphological alterations of unsporulated oocysts treated with *C. nucifera* extract

Pit on oocyst wall

Sporocyst shape variation

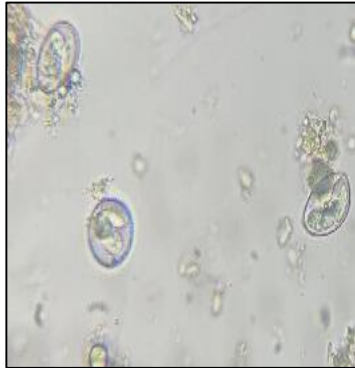
Compressed oocyst wall



Plate 24: Morphological alterations of unsporulated oocysts treated with

***A. hypogea* extract**

Damaged oocyst mass



Wrinkled oocyst



Leakage of oocyst mass



Fig. 1. Percentage yield of four plant extracts

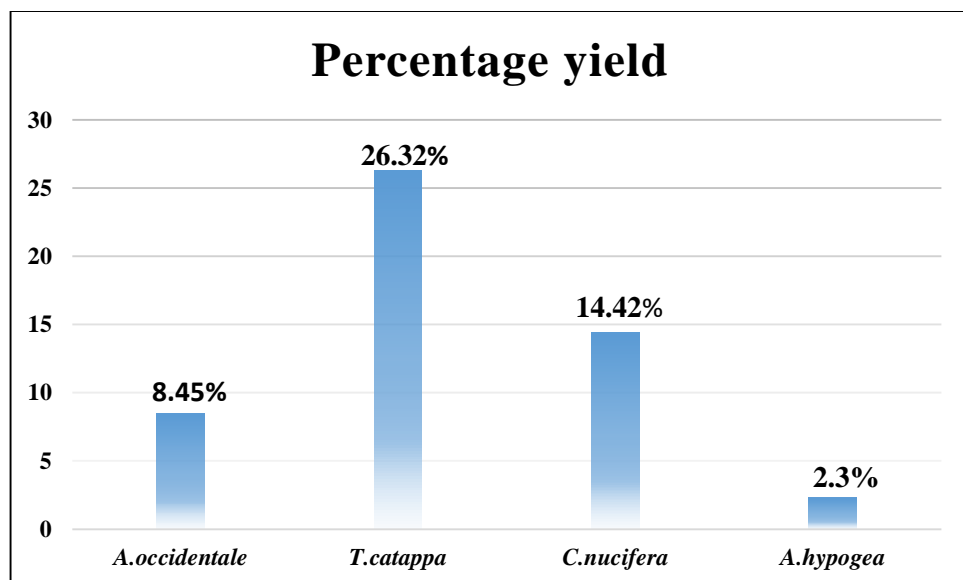


Fig. 2. Effect of methanolic extract on unsporulated oocysts of *Eimeria*

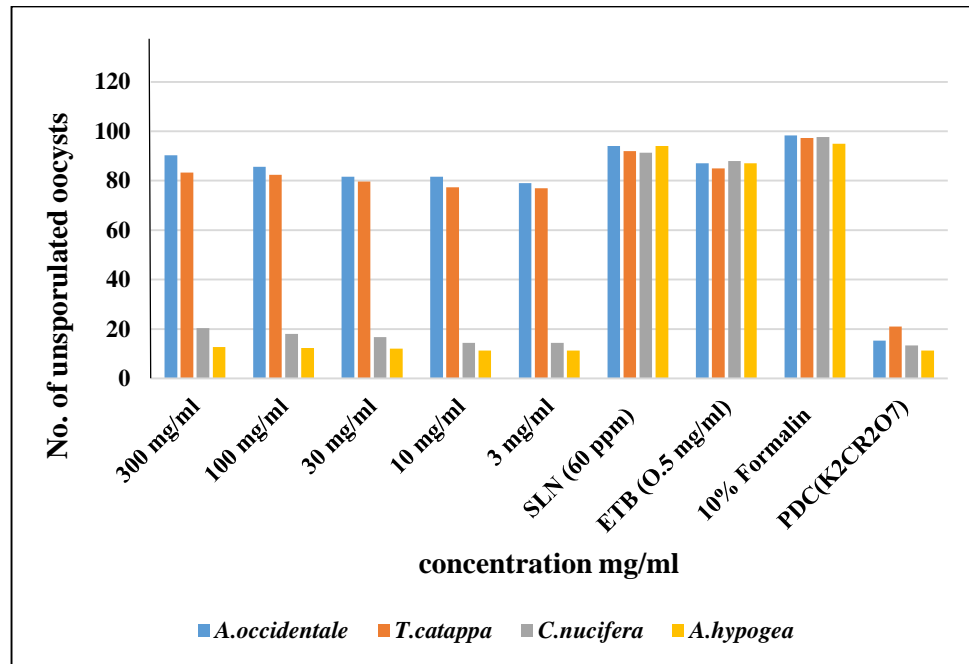


Fig. 3. Percentage efficacy of methanolic extracts using sporulation inhibition assay

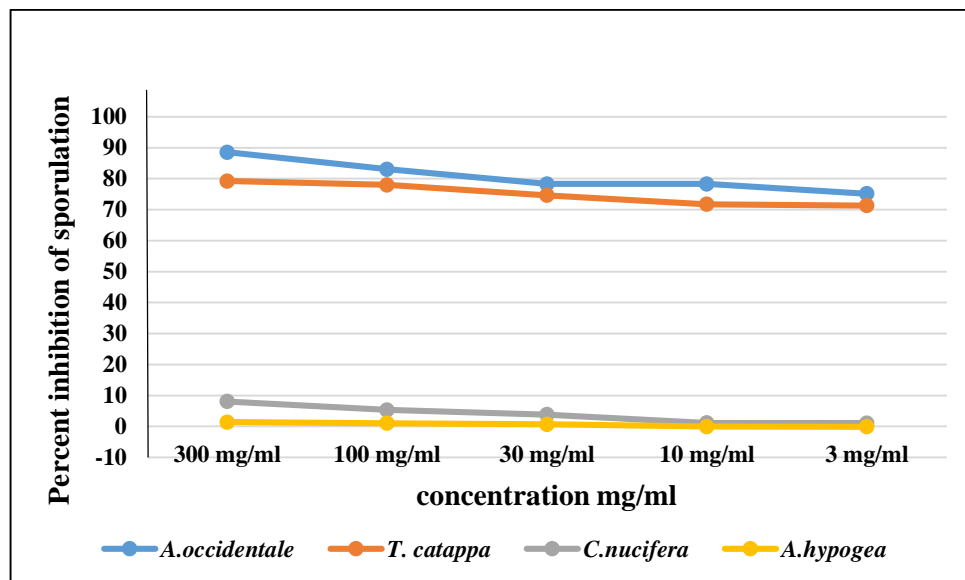


Fig. 4. Probit unsporulated oocysts x log concentration plot: SIA with methanolic extract of *A. occidentale*

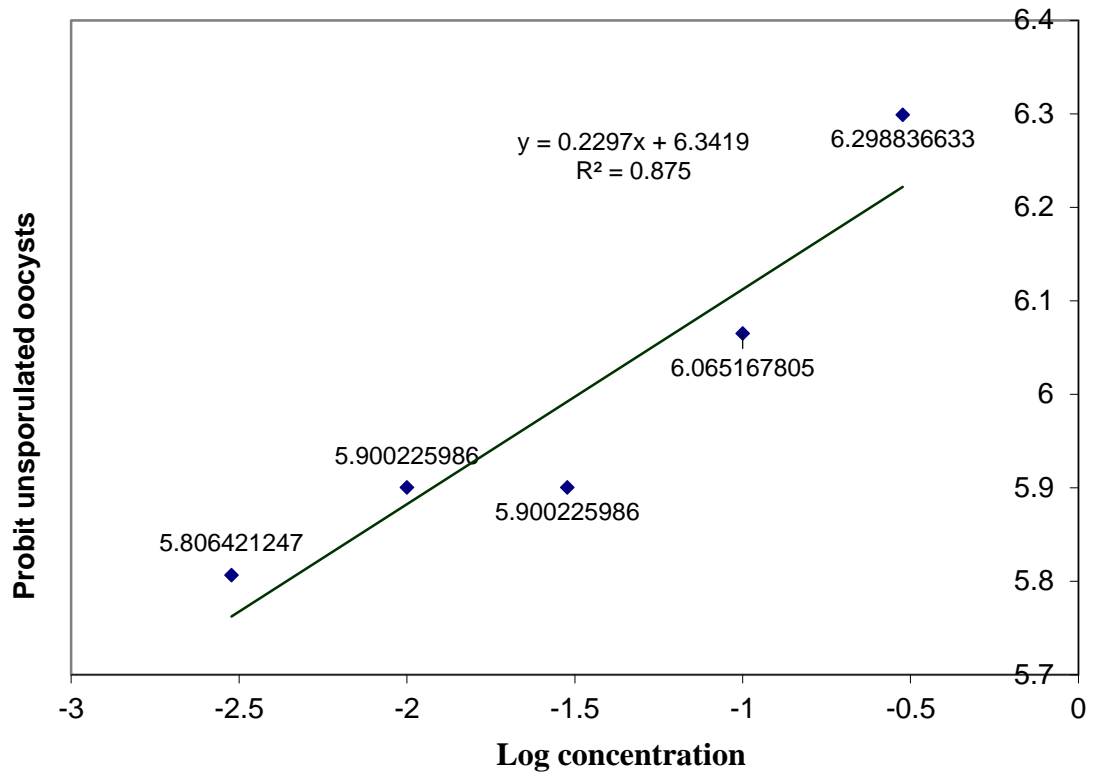


Fig. 5. Probit unsporulated oocysts x log concentration plot: SIA with methanolic extract of *T. catappa*

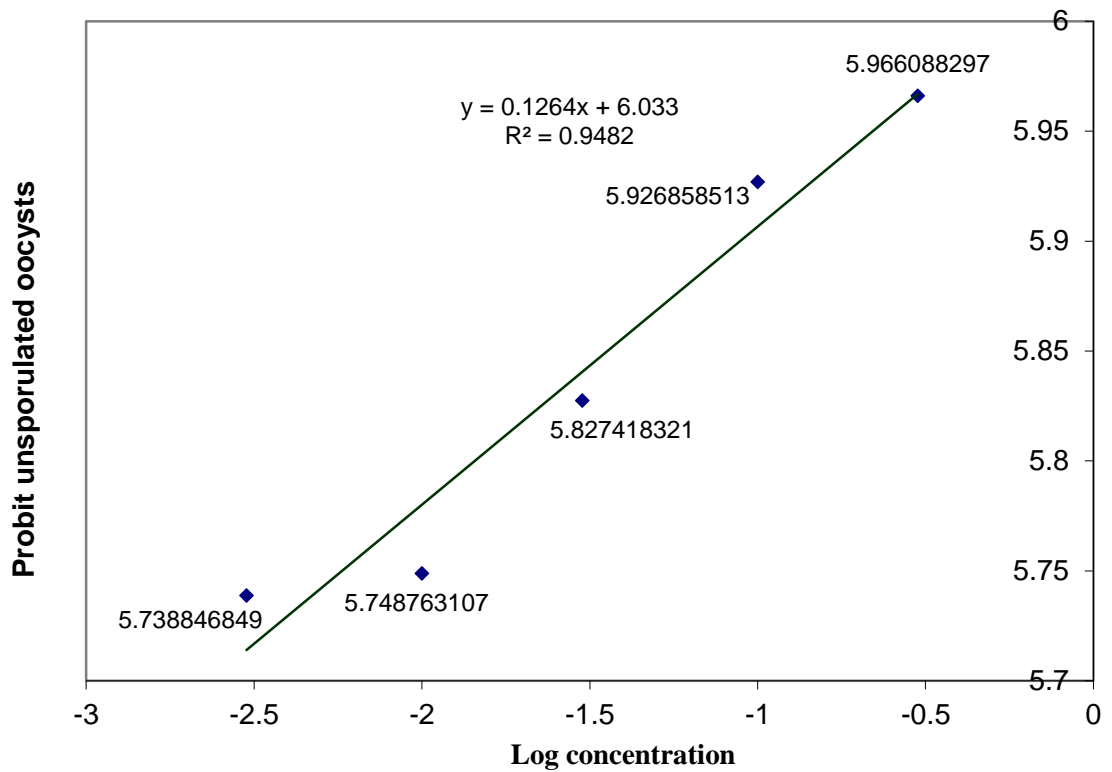


Table 4. Mean inhibition of sporulation \pm SEM of four methanolic extracts at different concentrations against *Eimeria* oocysts

Treatment \ Concentrations	<i>A. occidentale</i> **	<i>T. catappa</i> **	<i>C. nucifera</i> **	<i>A. hypogea</i> **
300 mg/ml **	88.58 \pm 0.39 ^{3bc}	79.24 \pm 0.83 ^{2bc}	8.07 \pm 2.34 ^{1a}	1.43 \pm 0.75 ^{1a}
100 mg/ml **	83.07 \pm 2.76 ^{2ab}	77.99 \pm 0.41 ^{2bc}	5.38 \pm 2.40 ^{1a}	1.05 \pm 1.64 ^{1a}
30 mg/ml **	78.34 \pm 2.58 ^{2a}	74.68 \pm 2.90 ^{2ab}	3.84 \pm 3.00 ^{1a}	0.68 \pm 1.72 ^{1a}
10 mg/ml **	78.34 \pm 4.84 ^{2a}	71.77 \pm 2.31 ^{2a}	1.15 \pm 3.35 ^{1a}	-0.07 \pm 1.36 ^{1a}
3 mg/ml **	75.20 \pm 4.47 ^{2a}	71.36 \pm 2.16 ^{2a}	1.15 \pm 2.78 ^{1a}	-0.07 \pm 1.36 ^{1a}
salinomycin (60 ppm) (positive control)	92.91 \pm 1.80 ^{1cd}	90.04 \pm 1.90 ^{1d}	89.99 \pm 1.54 ^{1b}	93.23 \pm 1.30 ^{1c}
ethopabate (positive control)	84.64 \pm 2.73 ^{1abc}	83.81 \pm 1.44 ^{1c}	86.15 \pm 1.99 ^{1b}	85.33 \pm 1.96 ^{1b}
10% formalin (positive control)	98.03 \pm 1.04 ^{1d}	96.68 \pm 1.81 ^{1e}	97.31 \pm 1.02 ^{1c}	94.36 \pm 1.30 ^{1c}
Potassium dichromate (K₂Cr₂O₇)	15.33 \pm 0.88 ^{1ab}	19.67 \pm 0.88 ^{1b}	13.33 \pm 1.76 ^{1a}	11.33 \pm 2.03 ^{1a}

Means with different superscript (roman number) within a row differ significantly (P<0.01)

Means with different superscript (alphabet) within a column differ significantly (P<0.01)

Table 5. ED₅₀ of methanolic extracts using SIA

Extract	ED ₅₀ (μg)	95%confidence limit		Regression equation $y = bx + a$	R ²
		Upper limit	Lower limit		
<i>A.occidentale</i>	1.1	11682.28	0.07	$Y=0.2297x+6.3419$	0.86
<i>T. catappa</i>	7.62	5.54E+13	9.02E-05	$Y=0.1264x+6.033$	0.95

CHAPTER-V

DISCUSSION

Coccidiosis is a major intestinal parasitic disease in chicken caused by intracellular apicomplexan protozoa *Eimeria*. Disease is characterized by diarrhoea, haemorrhage, poor growth, increased susceptibility to other disease agents and in severe cases, mortality. Annually, the poultry industry spends about £7.7 to £13.0 billion (at 2016 prices) on prophylaxis, treatment and production losses due to avian coccidiosis (Blake *et al.*, 2020). The most significant problem is the subclinical coccidiosis, which is characterized by suboptimal flock performance due to increased feed intake and reduced body weight gain (Remmal *et al.*, 2011) representing nearly three-quarters of the total economic costs. As first line of defence several chemicals and ionophore anticoccidial feed additives have been used widely since 1939 against these parasites (Nogueira *et al.*, 2009). This widespread use and abuse of anticoccidial drugs has resulted in development of resistance in *Eimeria* species (Abbas *et al.*, 2012). Fortunately, the immunity produced by infections with *Eimeria* spp. made vaccination an effective alternative to chemotherapy for control (Allen and Fetterer, 2002; Chapman *et al.*, 2005). But, these vaccines can trigger severe haemorrhagic reactions or malabsorptive coccidiosis where poor management affects flock's performance (Chapman *et al.*, 2002; Shirley *et al.*, 2005). In addition to this, drug residues concerning the consumers have directed research towards natural, safe and effective alternative strategies.

Herbal medicine is one of these alternatives to anticoccidial drugs, which is considered an attractive way against coccidiosis in compliance with the anticoccidial chemical-free regulations. Jang *et al.* (2007) studied the anticoccidial effects of green tea-based diets in chickens following oral infection with *E. maxima* and found that the green tea-fed chicken produced significantly reduced faecal oocysts when compared to the *E. maxima*-infected group fed standard diet. Similarly, (Molan and Faraj, 2015) studied the

effects of aqueous extracts from green tea on the sporulation of *Eimeria* species and found that addition of 10% and 25% (v/v) of tea extracts to the incubations containing unsporulated oocysts resulted in a significant reduction in sporulation rate. In addition, up to 30% of the oocysts recovered from incubations containing 25% of tea extract were with abnormal sporocysts. The anticoccidial properties of several natural herbal products (or their extracts) have been reported worldwide (Naidoo *et al.*, 2008; Anosa and Okoro, 2011; Habibi *et al.*, 2014; El Banna *et al.*, 2016; Muthamilselvan *et al.*, 2016; Pop *et al.*, 2019). Even, some of these herbal products are used in poultry diets due to their growth-promoting and natural immuno-stimulating effects. Agricultural by-products rich in phenolic and antioxidant chemicals could be used as useful additives in cattle feed (Castrica *et al.*, 2019). Supplementing natural feed additives with phenolic compounds may improve antioxidant, immune, antimicrobial, and overall production performance in poultry and swine (Mahfuz *et al.*, 2021).

Generally, the effect of herbal additives on avian coccidiosis is based on diminishing the oocyst output through inhibition of the invasion, replication and development of *Eimeria* species in the intestine of chickens; lowering oocyst counts due to the presence of phenolic compounds in herbal extracts which reacts with cytoplasmic membranes causing coccidial cell death; ameliorating the degree of intestinal lipid peroxidation; facilitating the repair of epithelial injuries; and decreasing the intestinal permeability induced by *Eimeria* species through the up regulation of epithelial turnover. Therefore, the present study was conducted to evaluate the anticoccidial effect of nut by-products namely nut peel of cashew (*Anacardium occidentale*), endocarp of green coconut (*Cocos nucifera*), hulls of almond (*Terminalia catappa*) and partially developed groundnut kernel (*Arachis hypogea*). This is the first study on the anticoccidial activity of claimed plant extracts on *Eimeria* species of poultry.

Methanol extracts are superior to other solvent extracts in their ability to scavenge

free radicals and demonstrate the highest antioxidant activity, for example almond hull extracts in methanol had a per cent polyphenolic yield of 0.956, ethanol had a yield of 0.675, and water had a yield of 0.942 (Pinelo *et al.*, 2004). Hence, methanolic extracts were prepared from claimed plants. The methanolic extracts of selected plant materials, displayed difference in per cent yield. The highest per cent yield was obtained for almond hulls (26.32%) and the lowest yield (2.3%) was recorded for partially developed ground nut kernels. The variation in the yield of extracts could be due to a difference in the chemical composition, with some plants containing active components that are more soluble in organic solvents.

Sporulation inhibition bioassay was used to evaluate the effect of these four methanolic extracts on the sporulation of *Eimeria* oocysts. However, most of the researchers explored *in vitro* effect by using cell culture method (Burt *et al.*, 2013) and effect on oocysts shedding after administration of anticoccidials (Zaman *et al.*, 2012). But, the assay employed in the present study is cost effective and time efficient. In addition, this method is equally valid for assessment of efficacy of inactivation measures performed for botanicals and synthetic agents to disinfect the environment (Zaman *et al.*, 2015).

The methanolic extracts of *A. occidentale* peel and *T. catappa* hulls showed significant ($P < 0.01$) inhibitory effect on sporulation of *Eimeria* oocysts (88.5 and 79.5% inhibition) at highest concentration of 300 mg/ml in comparison with negative control (potassium dichromate) with reference drugs (salinomycin, ethopabate and 10% formaline) being more effective than the plant extracts. The results were similar to previous studies conducted for other plants *in vivo* (Zaman *et al.*, 2012; Nghonjuyi *et al.*, 2015) and *in vitro* (Molan *et al.*, 2009; Khalafalla *et al.*, 2011; Habibi *et al.*, 2014) where extracts induced inhibition of oocysts sporulation *in vitro* at concentration lower than present concentration. Pine bark extract induced 86.4% inhibition in oocysts sporulation

at 1000 µg/ml concentration (Molan *et al.*, 2009). The methanolic extract of *Saccharum officinarum* at a concentration of 10% w/v induced 80% sporulation inhibition (Abbas *et al.*, 2015). The methanolic leaves extract of *Lannea schimperi* showed 98% sporulation inhibition against *E. tenella* oocysts at a concentration of 100 mg/ml (Mikail *et al.*, 2016). Contrary to the present observation, *Prangos ferulaceae* extracts at the end of 72 h induced only 29.69 % of sporulation inhibition (Habibi *et al.*, 2014) at higher concentration.

In the present study the inhibition of sporulation by the extracts was not dose dependent though many herbal extracts induced dose dependant inhibition in previous studies. Arlette *et al.*, (2019) observed dose dependent inhibition of oocysts sporulation ($P < 0.05$) using ethonolic and aqueous extract of *Ageratum conyzoides* and *Vernonia Amygdalina* on *E. tenella* oocysts. Though the phytochemicals in *C. nucifera* were found to be effective as antiulcerogenic, wound healing, antimicrobial, anti-inflammatory, anti-diabetic, anti-neoplastic, antiparasitic, insecticidal, leishmanicidal and antioxidant properties (Kannaian *et al.*, 2020), the methanolic extracts of endocarp of *C. nucifera* did not inhibit the sporulation of *Eimeria* oocysts. Similarly, the methanolic extracts of partially developed groundnut kernel did not inhibit the sporulation of *Eimeria* oocysts even though phytochemical constituents such as phytosterols, phenolics, stilbenes, arginine, resveratrol, luteolin and quercetin of *A. hypogea* elicited several pharmacological activities (Lopes *et al.*, 2011) like antimicrobial, antifungal, antiviral, antioxidant, anticancer and anti-inflammatory activities. The strong oocyst wall, might have acted as impediment, blocking the phytochemical components of these extracts to enter into the wall (Gadelhaq *et al.*, 2018).

In addition to inhibition of oocysts sporulation, extracts including methanolic extracts of partially developed groundnut kernel and endocarp of *C. nucifera* induced morphological abnormalities at all concentrations in treated oocysts with respect of oocyst walls (boat shaped, compressed and pitted appearance and rupture) and size

(reduced size). Morphological abnormalities were not seen in oocysts those recovered from the control incubations containing salinomycin, ethopabate and potassium dichromate. The abnormalities were identical to the findings of (Wondimu *et al.*, 2021), in which *Vernonia amygdalina*, *Croton macrostachyus* and *Azadirachta indica* extracts produced oocyst wall distortion at higher concentrations (100 mg/ml). While in another study, 1:100 dilution of Polioel 3 (mixed oil extract) revealed lowest rate of sporulation (64.4%) and the morphological alteration of oocysts when compared to the controls (Titilincu *et al.*, 2008). Molan *et al.* (2009) also observed aberrant sporocysts in oocysts subjected to 500 and 1000 g pine bark extract /ml.

It is hypothesized that the phytochemicals exhibit anti-sporulation effect by interfering in the physiological process necessary for sporulation process like preventing access of oxygen and inhibition of the enzyme responsible for sporulation due to the presence of secondary metabolites such as tannins, terpenoids, alkaloids and saponins. These polyphenolic compounds penetrate the oocyst wall and interfere with the development of sporocysts and sporozoites.

Feeding fresh pine needles and dry oak leaves (condensed tannins containing plants) in combination with lucerne chaff showed anticoccidial activities in goats as demonstrated by a sharp decrease in oocyst production (Hur *et al.*, 2005). Condensed tannins (CT) have been shown to inhibit endogenous enzyme activities (Oh and Hoff, 1986; Horigome *et al.*, 1988), and then it is possible that the tannins from *A. occidentale* nut peel and *T. catappa* hulls reduced the proportion of sporulation by inhibiting or inactivating the enzymes responsible for the sporulation process. Jones *et al.* (1994) suggested that CT may penetrate the cell wall of bacteria and cause a loss of intracellular components. In the present study, the tannins from *A. occidentale* nut peel and *T. catappa* hulls might have penetrated the wall of the oocyst and damaged the cytoplasm (sporont) as evidenced by the appearance of abnormal sporocysts in oocysts exposed to extracts.

The mechanism of action of the antimicrobial activity of terpenoids (Barre *et al.*, 1997; Amaral *et al.*, 1998) is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. The antimicrobial effects of alkaloids (Omulokoli *et al.*, 1997) such as berberine and harmaline (Hopp *et al.*, 1976) are attributed to their ability to intercalate with DNA (Phillipson *et al.*, 1987). Saponins inhibit the development of protozoa by interacting with the cholesterol present on the parasite cell membrane, thus resulting in parasite death (Wang *et al.*, 1998). Zaman *et al.* (2012) observed improved weight gains in the herbal complex medicated birds due to the growth-promoting effects of the saponin contents of the *Calotropis procera* plant, which is one of the plants in the herbal complex.

Thin layer chromatography analysis and qualitative phytochemical screening of extracts revealed presence of flavonoids, tannins, saponins, carbohydrates, triterpenoids and alkaloids that might be responsible for the anticoccidial effects noticed. Phytochemical analysis of almond hull extracts revealed the presence of phenolic compounds, flavonoids, and tannins, which were known for the radical scavenging activity (Kahlaoui *et al.*, 2019). *Anacardium occidentale* is a rich source of phenolic compounds and useful in the treatment of oxidative stress-related disorders (Ajileye *et al.*, 2015). Based on the concentration required to induce 50% sporulation inhibition (ED₅₀), *A. occidentale* nut peel induced 50% inhibition at lower concentration compared to *T. catappa* extract. The possible reason for potent anticoccidial activity of extracts of *A. occidentale* nut peel over *T. catappa* hulls extract might be due to the alkaloids that are exclusively present in it. Minor differences shown in the results of the plants may be attributable to various factors *i.e.*, ability of the phytochemicals in crossing the resistant oocyst wall and variation in molecular size, might be smaller in those herbs that hinders/ceased the sporulation process more significantly.

Conclusion

The studied herbal products may be used as a potential anticoccidial agent as they

are cheap, commonly available, and easy to use particularly for the resource poor farmers. Incorporation of these products in integrated coccidiosis management practices will add to the sustainability and thus, income of the farmers. However, large-scale controlled studies are recommended for standardization of the doses and applications of the product. Studies on fraction-based activity of formulation will be useful in identifying the active principles leading to development of a refined product with better anticoccidial efficacy.

CHAPTER-VI

SUMMARY

Coccidiosis constitutes a major health problem to the poultry industry and has primarily been controlled by the use of standard medication under field conditions in spite of limitations like drug resistance and other concerns in relation to food chain contamination. As an alternative, the use of plants and their products as immunomodulators and therapeutics have traditionally been used. Hence, the present study was conducted to evaluate the effect of methanolic extracts from cashew (*Anacardium occidentale*) nut peel, green coconut (*Cocos nucifera*) endocarp, almond (*Terminalia catappa*) hulls and partially developed groundnut (*Arachis hypogea*) kernel on sporulation and morphology of oocysts of *Eimeria* species of poultry. Methanolic extraction was conducted by mixing 20 g of the dried and powdered plant material with 200 ml of 100% methanol in a conical flask. The solvent was evaporated in a water bath at 50°C and the dried extracts were suspended in dimethyl sulfoxide for obtaining a final concentration 500-1000 mg/ml of stock solution. The methanolic extracts of selected plant materials, showed difference in per cent yield. The yield of *T. catappa* hulls, *C. nucifera* endocarp, *A. occidentale* nut peel and partially developed *A. hypogea* was 26.32, 14.42, 8.45 and 2.3 per cent, respectively. Inhibition of sporulation was criterion for evaluation of the efficacy of extracts.

Sporulation inhibition bioassay was used to evaluate the effect of four extracts on the sporulation of *Eimeria* oocysts. For this purpose faecal samples were collected from 492 birds from poultry farms and slaughter houses in and around Krishna district, Andhra Pradesh; out of which 385 were found positive for oocysts of *Eimeria* species on microscopic examination with a prevalence of 78.25 per cent. Further morphometric analysis of unsporulated oocysts revealed the presence of *E. tenella*, *E. necatrix*, *E. maxima*, *E. brunette* and *E. acervulina* oocysts. In sporulation inhibition assay,

unsporulated oocysts were exposed to five (300, 100, 30, 10, and 3 mg/ml) concentrations of each extract in phosphate buffer solution in Petri dishes and cell-culture plates while salinomycin, ethopabate, formalin (10%) (positive control) and potassium dichromate solution (negative control) served as control groups. The Petri dishes and cell-culture plates were partially covered to allow the passage of oxygen and incubated at 25-29 °C for 48 h, providing 80% humidity. The sporulation of the oocyst was confirmed by examining sporocysts under microscope at 40x at the end of 48 h of exposure.

Results showed anticoccidial activity of extracts against *Eimeria* species as proved by its ability to inhibit the sporulation of the oocysts under laboratory conditions. At the end of 48 h of exposure, among four extracts, the methalolic extracts of *A. occidentale* nut peel and *T. catappa* hulls showed significant ($P<0.01$) inhibitory effect (88.58 ± 0.39 and $79.24\pm 0.83\%$, respectively) at highest concentration of 300 mg/ml on sporulation of *Eimeria* oocysts in comparison with negative control, with reference drugs *i.e.*, salinomycin, ethopabate and formalin (10%) (92.91 ± 1.80 , 84.64 ± 2.73 and $98.03\pm 1.04\%$, respectively) being more effective than the plant extracts. But the inhibition of sporulation was not dose dependent for *A. occidentale* nut peel and *T. catappa* hulls extracts as there was no significant variation in mean inhibition at different dose levels for each type of extract. Independent comparisons revealed that at the concentration of 100 mg/ml or higher, the percentage inhibition of *A. occidentale* nut peel was significantly higher ($P<0.05$) than that of *T. catappa* hulls. Methanolic extracts of *A. occidentale* nut peel induced nearly 90% per cent sporulation inhibition at 300 mg/ml. The effective doses required to induce 50% (ED_{50}) inhibition of sporulation was calculated by probit analysis. Extracts of *A. occidentale* nut peel induced 50% inhibition at lower concentration ($1.099\ \mu\text{g/ml}$) compared to *T. catappa* extract ($7.627\ \mu\text{g/ml}$). At highest concentration (300 mg/ml) the rate of sporulation inhibition by *C. nucifera* endocarp and partially developed *A. hypogea* kernels was 8.07 ± 2.34 and 1.43 ± 0.75 per

cent, respectively, which was inappreciable in comparison with reference drugs salinomycin, ethopabate and formalin (10%). All four extracts damaged the normal morphology and shape of oocysts of *Eimeria* species.

Thin layer chromatography analysis and qualitative phytochemical screening of extracts revealed presence of flavonoids, tannins, saponins, carbohydrates, triterpenoids and alkaloids that might be responsible for the anticoccidial effects noticed. In conclusion, this *in vitro* study suggests that *A. occidentale* nut peel and *T. catappa* hulls have promising anticoccidial effects. Studies on identification of the active principles with better anticoccidial efficacy in each plant are warranted. Nonetheless, further *in vivo* studies through challenging broiler chicken using the sporulated oocysts treated with *A. occidentale* nut peel and *T. catappa* hulls extracts is suggested.

CHAPTER – VII

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