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Herbal Antibiogram and Antisporulation: In Vitro Assessment on Bacterial Isolates and Eimeria oocysts from Rural Poultry in South Andaman

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Abstract

In-vitro work was designed to determine antimicrobial and anti-sporulation effect of Zingiber spectabile, Piper betle, Cissus quadrangularis, Costuspictus and Centella asiatica extracts on cloacal isolates and Eimeria oocysts from Vanaraja birds. Antibiogram in terms of zone of inhibition (ZI) of methanolic extracts of herbal extracts at concentrations (µg) against salmonella and E. coli isolates were performed. The percentage of sporulation inhibition (SI) of herbal extracts was estimated on oocysts collected from naturally coccidiosis infected birds. Extracts of Zingeber spectabile (510 µg) and Piper betle (557 µg) exhibited significantly highest antibacterial activity (15.92 mm) against E. coli isolates and against the Salmonella isolates it was 16.6 mm with Zingeber spectabile (587 µg) and Piper betle (534 µg). The extracts of Zingiber spectabile(root) and Piperbbetle (leaf) inhibited sporulation of oocysts significantly higher (>80%). The anti-bacterial properties and pronounced anti-sporulation activity on oocysts of methanolic extracts of these medicinal plants have revealed that they could be further studied and explored for feed and water supplements as anti-coccidial in poultry.

Keywords: Antibiogram, Antisporulation, Coccidialoocysts, Herbal Extracts, Rural Poultry, A&N Islands

Introduction

The challenge to tap maximum production from the poultry is the control and prevention of infectious diseases such as colibacillosis, salmonellosis and coccidiosis which are accountable for major economic losses (Chenniappan *et al*., 2020; Hafez, 2008). Emergence of antibiotic resistant organisms (Awasthi, *et al.*, 2019) and drug resistant parasites (Long & Reid, 2012) insists alternate strategy on medicinal plants for antimicrobial (Awasthi, et al., 2019) and anticoccidial agents (Augustin *et al*., 2018). The primary step in the development of phyto-chemotherapy is in-*vitro* antibacterial activity assay (Zarchil and Babaei, 2006) of medicinal plants. Analysis of medicinal plants for both antimicrobial and anticoccidial properties are reported (Augustin *et al*.,2018; Ebrahimabadi *et al*., 2010). Andaman & Nicobar Islands is home to endemic and rich diversity of medicinal plants. The aboriginals of the Island use a variety of endemic plants for primary health care (Abirami *et al*.,2017; Sharma *et al*., 2018). Several *in-vitro* studies have been carried out to assess the antimicrobial and anti-fungal properties of medicinal plants (Sunder *et al*. 2012; Sunder *et al*., 2016; Sujatha *et al*., 2017a, b & c; Sujatha and Jai Sunder, 2020). In the present study, the medicinal plants viz., *Zingiber spectabile, Piper betle, Cissus quadrangularis, Costus pictus* and *Centella asiatica* were used for *in-vitro* screening of their both anti-microbial and anti-coccidial efficacy. Anti-microbial efficacy of *Zingiber spectabile* (Chenniappan *et al*., 2020), *Piper betle* (Bangash *et al*., 2012) and *Cissus quadrangularis* (Sivasothy *et al*., 2013) have been reported. Similarly, a few *in-vivo* studies have been carried out on reduction of faecal oocysts count by feeding *Zingiber officinale* and *Piper nigram/guineense* (Eze *et al*., 2020). *Zingiber officinale* and *Cissus quadrangularis,* (Muthamilselvan *et al*., 2016; ShishayMarkos, 2019) have been documented for the treatment of coccidiosis. However, the iranti-sporulation efficacy on *Eimeria* oocystsis yet to be studied. Hence, this *in-vitro* work was designed to determine antimicrobial and anti-sporulation effect of *Zingiber spectabile, Piper betle, Cissus quadrangularis, Costus pictus* and *Centella asiatica* extracts on isolates and *Eimeria* oocysts from Vanaraja birds.

Materials and Methods

Extraction of Medicinal Plants

Methanolic extracts of medicinal plants were prepared asper the method described by S´anchez *et al*. (2010). Plants such as *Zingeber spectabile, Cissus quadrangularis, Costus pictus, Centella asiatica* and *Piper betle* were collected from South Andaman and processed for methanol extraction. Collected samples were washed with tap water and powdered after drying under shadow. Samples were then soaked with periodical manual shaking in methanol at the ratio of 1:10 dilution for 3 days. After the period, it was filtered and kept at 40 – 50^0 C in water bath until methanol is completely evaporated. Subsequently, methanol extracts of those medicinal plants were dissolved in dimethyl sulfoxide (DMSO (10% w/v) at the ratio of 1: 5 (Mishra and Padhy, 2013) to arrive at final concentration of 50 µg/µl. The diluted extracts were stored at 4°C till further usage for the work.

Isolation of Organisms and Herbal Antibiogram

A total of 30 cloacal swab samples were collected from diarrheic Vanaraja poultry of free range systemin villages *viz*., Chouldhari (11.6405° N, 92.6611° E), Rangachang (11.5840° N, 92.7350° E) and Siphighat (11.48326° N, 92.6077°) of South Andaman. The swabs were inoculated in nutrient broth and incubated at 37°C overnight. The broth culture was streaked on next day to Eosine Methylene Blue (EMB) and MacConkeyagar plates and incubated at 37°C overnight. *E. coli* and *Salmonella* isolates were identified based on colonial morphology on specific agar and biochemical tests (HiMedia, Mumbai, India) as per standard method (Cappuccino and Sherman, 2001;Cheesebrough, 2006). Antibiogram of herbal extracts against isolates were performed using conventional Kirby-Bauer's disc diffusion method (Bauer *et al*., 1966) with Mueller–Hinton (MH) agar medium (HiMedia, Mumbai, India). Six mm filter paper discs were prepared and autoclaved (121°C and 15lbpressure) which were soaked with the extracts by dispensing the extracts on the discs up to the maximum absorption capacity of the discs. The concentration of extracts in each disc was calculated. Gentamicin (G-30µg) disc was used as a positive control and DMSO was added in a separate disc as a negative control. The extracts impregnated discs were placed on the plates which were then incubated for 24 h at 37°C. Zone of Inhibition (mm)of each extract was noted against isolates (Growther *et al*., 2012) and was interpreted with reference to Harun *et al*. (2016).

Collection of Eimeria oocysts and Herbal Anti-Sporulation

Intestinal and caecal samples from naturally coccidiosis affected Vanaraja poultry were obtained from poultry farms in Garacharma and Chouldhari (11.6405° N, 92.6611° E), South Andaman. The caecum & intestine were cut and placed in sample cover. The samples of caecum and intestinal contents of coccidiosis were processed and sporulation counting was done using modified M'C Master technique (Cedric *et al*., 2017). One gram of samples (caecum & intestine) was taken and 7.5 ml of distilled water was added. It was ground through mortar pastel and mixed well. The sample was then filtered using mesh. The mixture was prepared with the filtrate (0.5 ml) and 0.5 ml of Syther's sucrose solution. The mixture was placed on the slide with the help of syringe. The unsporulated oocysts were counted under microscope (10X). The *in-vitro* study on effect of medicinal plants extract on sporulation of oocysts comprised of two treatments. The treatment one was prepared with a mixture of 100µl of unsporulated oocysts ($1X10^3$) plus 100 µl potassium dichromate + 100 µl of medicinal plant extract in a vial and covered with aluminium foil made with pores and incubated for 48 h at 4^0C. The treatment two (control) was composed of 100µl of unsporulated oocysts ($1X10^3$) + 100 µl potassium dichromate + 100µl of distilled water in vial. All the treatments were incubated at room temperature for 48 h and were then stored at 4^0C. The observations of sporulation and unsporulation of oocysts were made under microscope at 10X as per the method (Molan *et al*., 2009) to be used for *in-vitro* experiment. The percentage of sporulation was estimated by counting the number of sporulated oocysts in a total of 100 oocysts. The percentage of sporulation inhibition (SI) of oocysts was calculated as per the following formula (You, 2014).

SI%=Sporulation of control-Sporulation of treated/sporulation of Control×100

Statistical Analysis

Data were expressed as mean ± S.E.M. Statistical reading and comparison among the group was performed by one-way analysis of variance (ANOVA) by least significant differences (LSD) test with a p value ≤ 0.05 was considered significant (Bahndry *et al.,* 2012).

Results and Discussion

Herbal Antibiogram Against Isolated Organisms

A total of 15 (50%) isolates of *Salmonella* and 18 (60%) *E.coli* was identified based on their colony characteristics and biochemical profiles. Antibacterial activity (Table 1) of the tested extracts of plants on *E.coli* and *Salmonella spp.* varied significantly. Extracts of *Zingeber spectabile* (510 µg) and *Piper betle* (557 µg) exhibited significantly highest ($p<0.05$) antibacterial activity with an average ZI of 15.92 mm against *E. coli* isolates. Extract of *Centella asiatica* (517 µg) recorded statistically medium ZI of 12.0 mm. The trend of ZI against *Salmonella* isolates was similar as that of *E. coli* isolates. Significantly highest ZI (16.6 mm) was reported with *Zingeber spectabile* (587 µg) and *Piper betle* (534 µg) against the *Salmonella* isolates. Extract of *Centella asiatica* (526 µg) recorded statistically medium ZI of 11.0 mm against *Salmonella* isolates. *Cissus quadrangularis* and *Costus pictus* recorded significantly lowest ZI of 6 mm against both *E. coli* and *Salmonella* isolates.

Medium antibacterial activity has been exhibited by *Zingeber spectabile* and *Piper betle* as per classification of antibiogram of medicinal plants (Harun *et al*., 2016) as given in the foot note of Table 1. According to classification of Kirby-Bauer (2017), the antibacterial activity of *Zingiber spectabile* and *Piper betle* was corresponding to intermediate susceptibility of antibiotics such as Ampicillin (>14 mm), Cephalothin (15-16 mm), Chloramphenicol (13-17 mm), Clindamycin (15-20 mm), Erythromycin (14-22mm), Gentamicin (13-14 mm), Kanamycin (14-17 mm), Lincomycin (10-14 mm), Methicillin (10-13 mm), Neomycin, Sulfonamides (13-16 mm), Tetracycline (15-18 mm), trimethoprim (11-15mm), Nalidixic acid (14-18 mm) and fluroquinolones (15-17mm). Reports suggests that the presence of phenolic secondary metabolites in the rhizomes of *Zingeber spectabile* (Sivasothy *et al*.,2013; Sampate *et al*., 2019) justified the antibacterial activity of extracts of root of *Zingiber spectabile* as shown in the present study.

Table 1: Antibacterial activity of methanolic extracts of medicinal plants

S. No.	Plant name	Antimicrobial activity against *E. coli*		Antimicrobial activity against *Salmonella spp*	
		Zone of Inhibition (mm)*	Minimum Inhibitory concentration (µg)	Zone of Inhibition (mm)*	Minimum Inhibitory concentration (µg)
1.	*Zingiber spectabile* (root)	15.7±0.23[a]	510	16.7±0.2[a]	587
2.	*Cissus quadrangularis* (leaves)	6.0±0.31[c]	560	6.0±0.31[c]	590
3.	*Costuspictus* (leaves)	6.0±0.42[c]	550	6.0±0.42[c]	500
4.	*Centella asiatica* (leaves)	12.0±0.29[b]	517	11.9±0.13[b]	526
5.	*Piper betle* (leaves)	16.14±0.51[a]	557	16.5±0.32[a]	534
6.	DMSO (*Negative control)*	5			
7.	Gentamicin (30 µg) (*Positive controls)*	21			

*Data are presented as mean of measurement of zone of inhibition (ZI) of three replicates measured in mm.They have then categorizedZI 0-6 mm: no activity; 7-10 mm: weak inhibition; 11-15 mm: moderate inhibition; more than 16 mm: strongly inhibited (Harunet al., 2016); *Mean±SE having different superscript differ significantly (p<0.05)*

In line with the results obtained from this study, earlier studies (Jayaprakasha *et al*., 2006) have also reported higher antibacterial activity of *Zingiber spectabile* due to its abundant curcuminoids content. The permeability of the bacterial cells to the tested compounds is one of the determining factors of their antibacterial activity. The presence of adiene ketone system provides lipophilicity to the curcuminoids and thus enhances penetration into target organisms (Jayaprakasha *et al*., 2006). In line with the results obtained from this study, earlier studies (Jayaprakasha *et al*., 2006) have also reported higher antibacterial activity of *Zingiber spectabile* due to its abundant curcuminoids content. The present results therefore highlighted the remarkable broad-spectrum effects of *Zingiber spectabile* in inhibiting Gram-negative food-borne bacteria. Result of the *Piper betle*is in agreement with report (Bangash *et al*., 2012) on its higher activity against *E. coli* and *Salmonella* with ZI of 12mm and 23mm respectively. Similarly, methanolic extracts of *Piper betle* had also been reported as effective against *E. coli* and *Salmonella* (Suppakul *et al.,* 2006; Sharma and Khan, 2009; Syahidahn *et al*., 2017). Wide range of secondary metabolites such as hydroxychavicol and eugenol have been studied in *Piper betle* plant leaves attribute to antibacterial properties and *Piper bêtel* had been conventionally used as antibacterial agents (Fathilah *et al*., 2010; Atiya *et al*., 2018; Syahidah *et al*., 2017). The phenolic nature of constituents of *Piper betel* leaf are responsible for inhibiting bacterial growth (Bangash *et al*., 2012). As per Harun *et al*. (2016), extract of *Centella asiatica* had weak inhibition against both *E. coli* and *Salmonella* isolates. However, various reports also revealed that methanolic extract of *centella asiatica* has significant and higher rate of antimicrobial activities against various bacteria including *E. coli* and *Samonellasps*as it contains most active phytochemicals (Zaidan *et al*., 2005; Perumalsamy *et al*., 2011; Soyingbe *et al*., 2018). Similarly, the methanolic extracts of *Cissus quadrangularis* and *Costuspictus* had inhibition zones of 10 mm against *Salmonella* sp(Costa *et al*, 2008 ;Chenniappan *et al*., 2020). The presence of multi various bioactive compounds present in *Cissus quadrangularis* and *Costus pictus* has exerted antimicrobial activities.

Herbal Coccidiostatic Effects Against Oocysts

Efficacy of medicinal plant extracts on sporulation of intestinal and ceacal coccidial oocysts showed significant variations (Table 2). The extracts of *Zingiber spectabile* (root) and *Piper betle* (leaf) inhibited sporulation of oocysts significantly higher (>80%) as compared to control and other treatments. The *Piper betle* (leaf) inhibited more than 80% of sporulation of caecal oocysts and 70% of sporulation was inhibited by *Zingiber spectabile* (root). Extracts of *Zingiber spectabile* (leaf) and *Centella asiatica* showed statistically similar sporulation inhibitory action (>50%) on both intestinal and ceacal oocysts. Extracts of *Zingiber spectabile* (stem) showed more than 70% inhibitory action on intestinal oocysts and its inhibitory level on ceacal oocysts is less. The extracts of Cissus quadrangular is showed significantly less sporulation inhibitory action against both intestinal and caecal coccidial oocysts. The control group with potassium dichromate showed statistically lowest sporulation inhibition of both intestinal and caecal oocysts.

Table 2: Effect of medicinal plant extracts on sporulation inhibition % (Mean ± S. E) of intestinal and ceacal coccidialoocysts

Sample	Treatment	Sporulation Inhibition %**
Intestinal Oocysts	*Zingiber spectabile(leaf)*	58.21±2.36[e]
	Zingiber spectabile(Stem)	78.95±1.42[d]
	Zingiber spectabile(Root)	85.00±1.78[a]
	Control (Potassium dichromate)	12.50±0.59[g]
	Cissus quadrangularis	44.44±2.38[f]
	Centella asiatica	61.96±1.62 [e]
	Piper betle	84.16±3.67 [a]
CaecalOocysts	*Zingiber spectabile(leaf)*	60.00±3.24[c]
	Zingiber spectabile(Stem)	63.64±2.82[c]
	Zingiber spectabile(Root)	71.43±2.5[b]
	Control (Potassium dichromate)	11.95±0.73[e]
	Cissus quadrangularis	45.78±1.28[d]
	Centella asiatica	64.29±4.17[c]
	Piper betle	87.37±1.68[a]

***Columns having (Mean ± S. E) with different superscripts vary significantly at P≤0.001*

Similar to present *in-vitro* study on sporulation of *Eimeria* oocysts, Molan *et al*. (2009) also observed *in-vitro* sporulation inhibition with aqueous extracts of pine bark (*Pinus radiata*) and *P. guajava (Cedric et al.,* 2017). The *Piper betle* has been reported as anthelmintic (Shah *et al*., 2016) herb. Phytocompounds in *Piper* with proven suppressive action on *Eimeria* species have also been reported by Muthamilselvan *et al*. (2016) which might be attributed for the present result. The present *in-vitro* result of reduction in coccidial oocysts count by *Zingiber spectabile* technically supports the observation of Hayajneh (2017) that Z*ingiber officinale* controlled coccidiosis in broilers, although it is different species of *Zingiber*. The result of present study provides scientific attribute to the documentation of Shishay Markos (2019) that the crushed leaf of *Cissus quandrangularis* and crushed rhizome of *Zingiber officinale* are given to poultry as water additives to treat coccidiosis in poultry in Western Zone of Tigray, Northern Ethiopia. Administering aqueous extracts of *Zingiber officinale* and *Piper guineense* also reduced significantly the *Eimeria* oocysts counts in broilers (Eze *et al.,* 2020). Ginger as feed additive also reduced faecal oocysts count on 11th day post infection (Ali *et al.,* 2019). Other reports also confirmed the presence of alkaloids, saponins, tannins, flavonoids, phenolics and glycosides in aqueous extract of *Zingiber sps* and *Piper sps* (Ahmador *et al*., 2016; Iheukwumere *et al*., 2017) could be responsible (Eze *et al.,* 2020). *C. quadrangularis* is rich in polyphenolic and flavonoid content. *C. quadrangularis* also showed promising immune modulatory potential (Yadav *et al*., 2020) that might have caused reduction in oocysts count in the present study. The bioactive compounds of ginger viz., gingerdiol, shogaols, gingerol, gingerdione and other phenolics possessing antioxidant properties (Khan *et al*., 2012) might be accountable for coccidio static effect in the broiler. The detailed mode of action of these phytocomponents has been reported by Ahmed *et al*., 2018 that saponin bind to the sterol molecules present on the cell membrane of the parasites and bring on cytoplasmic lysis; tannins and phenolic inactivate the endogenous enzymes responsible for the sporulation process through penetrating into the wall of the oocyst and damaging the cytoplasm; flavonoids stimulates release of reactive nitrogen species (RNS) and reactive oxygen species (ROS) and causes oxidase stress leading to the death of the Eimeria sporozoites. They can prevent the proliferation of *Eimeria* species, improve the beneficial bacterial population and boost immunity that in turn control *Eimeria* infection of the gut (Muthamilselvan *et al*., 2016). The anticoccidial effects of medicinal plants are generally connected with active compounds present in plants which enhance immunity, antioxidant status, reduce intestinal inflammation and the parasitic load (Tanweer *et al*., 2014; Abudabos *et al*., 2016; Tehseen *et al*., 2016; Raza *et al*., 2016). Other authors have also attributed the anticoccidial activity of herbal extracts to its immune stimulant activity (Kim *et al*., 2013; Pourali *et al*., 2014; Khalil *et al*., 2015). Hence, the immune enhancing, anti-oxidant and anti-inflammatory properties of Z*ingeber sps* (Shengying *et al*., 2019), *Centella asiatica* and *Cissus quadrangularis* might also have resulted in their anti-coccidial effect. Eimeria species decrease in population as age advances due to increase in gut-associated T cells, macrophages and other immune cells which act against Eimeria species and causes coccidial clearance while coccidiosisisis frequently encountering young birds which are in developmental stage of immune system and whereby these immune enhancing medicinal plants become responsible for coccidiostatic effects (Muthamilselvan *et al*., 2016) during growing stage of poultry.

Conclusion

A more detailed systemic study is required to extrapolate the present results. However, this study revealed that methanolic extracts of Z*ingeber spectabile and Piper betle* have anti-bacterial properties against *E. coli* and *Salmonella* of poultry and could be further explored for their anti-microbial properties. Methanolic extracts of Z*ingeber spectabile, Piper betle, Centella asiatica* and *Cissus quadrangularis* have shown pronounced anti-sporulation activity on oocysts and thus they could be further studied and explored for feed and water supplements as anticoccidial in poultry.

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Conflict of Interests

There is no conflict of interest.

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