Indian J Anim Health (2022), 61(2): 285- 293
DOI: https://doi.org/10.36062/ijah.2022.04322

Research Article

Substitution of antibiotic growth promoter with locally available plant derivatives: *In-vitro* study

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Abstract

The use of antibiotic growth promoters in poultry diets as feed additives poses risks due to the generation of antimicrobial resistance among the commensal or pathogens and antibiotic residues in tissues. Phytogenic feed additives are a class of growth promoters, originating primarily from herbs, spices and their products, which have gained widespread interest in the feed industry in recent years. The present study was designed to evaluate the *in-vitro* antibacterial effects of aqueous-methanolic extract of six locally available herbs or their parts viz. *Ocimum sanctum* Linn. (Tulsi) leaf, *Nyctanthes arbor-tristis* (Seuli) leaf, *Solanum torvum* (wild eggplant) leaf, *Bryophyllum pinnatum* (Patharkuchi) and *Spondia dulchis* (Amra) fruit and *Punica granatum* (Pomegranate) peel against ESBL-producing *E. coli* and *Salmonella* isolated from poultry. *Ocimum sanctum* Linn. (Tulsi, 50 mg/mL), *Nyctanthes arbor-tristis* (Seuli, 50 mg/mL) and *Punica granarum* (Pomegranate, 50 mg/mL) were found most effective against ESBL-producing *E. coli* and *Salmonella*, which produced higher zone of inhibition than the control antibiotic. Minimum inhibitory concentration of the plant extracts ranged from 0.56 mg/mL to 1.20 mg/mL. *Ocimum sanctum* Linn. leaf extract produced lowest MIC value (0.56 mg/mL) against ESBL-producing *E. coli* and *Salmonella* among the studied plant extracts.

Key words: Antibiotic growth promoter, ESBL, MIC, *Ocimum sanctum*, poultry

Highlights

- *Ocimum sanctum* L., *Nyctanthes arbor-tristis*, *Punica granarum* were found effective against ESBL-producing bacteria.
- Minimum inhibitory concentration of the plant extracts ranged from 0.56 mg/mL to 1.20 mg/mL.
- *Ocimum sanctum* L. leaf extract had lowest MIC.

INTRODUCTION

Since the initiation of the use of antibiotic growth promoters (AGP) around 70 years ago, AGP at sub-therapeutic levels have been used as growth promoters in poultry diets (Kumar *et al.*, 2017). Use of AGP in poultry diets as feed additives poses risks due to cross-antibiotic resistance amongst pathogens and antibiotic residues in tissues (Schwarz *et al.*, 2001). The World Health Organization (WHO) recommended that AGP should be phased out and replaced by alternative growth promoters (Bywater, 2005). The use of most AGP has been banned in many countries, especially in the European Union, since 2006. The ban on using AGP in poultry diets have resulted in an intensified search for alternative natural growth promoters to sustain growth and laying performance of chickens.

Phytogenic feed additives (PFA) are a class of growth promoters, originating primarily from herbs, spices and their products, which have gained widespread interest in the feed industry in recent years (Wenk, 2003). Aromatic plants and essential oils (EO) extracted from the plants have become more important because of their antimicrobial actions (Wenk, 2003; Patra, 2012) and stimulating effects on

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the digestive systems of animals (Wenk, 2003; Jang *et al.*, 2004). The availability of large numbers of essential nutrients and a variety of pharmacologically active substances makes them suitable for use in poultry diets as ingredients as well as additives. The antimicrobial effects of PFA are primarily attributed to their phenolic components and their action on pathogenic cells (Burt, 2004; Si *et al.*, 2006). An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Sikkema *et al.*, 1994). Many of plant extracts such as neem (*Azadirachta indica*) oil, clove (*Syzygium aromaticum*) oil, karanga (*Pterocarpus marsupium*) oil, tulsi (*Ocimum sanctum* Linn.) oil, cashew (*Anacardium occidentale*) shell oil, henna (*Lawsonia inermis*), and prickly chaff (*Achyranthes aspera*) leaves contain compounds like phenolic, terpenoids, flavonoids, alkaloids, polyethylenes, polypeptides act as antimicrobials (Ravindra and Babu, 2016).

The *Ocimum sanctum* Linn. (OS; Tulsi) is an important herb of Indian traditional systems of medicine which has antioxidant, antibiotic, antiatherogenic, immunomodulatory, anti-inflammatory, analgesic, antiulcer, chemopreventive and antipyretic properties (Singh *et al.*, 2007). *Nyctanthes arbor-tristis (NA),* [Harsingar/Parijat in Hindi and Seuli in Bengali], is an important member of traditional medicine, with a diverse spectrum of medicinal properties, such as being anti-helminthic, antimicrobial, antiviral, anti-leishmania, anti-allergic, anti-diabetic and anti-cancerous (Agrawal and Pal, 2013). *Punica granatum* Linn. (PG) is locally named as pomegranate is a plant fruit that belongs to the *Punicaceae* (family). PG has bioactive components that can be used for the treatment of the dental diseases, cardiovascular diseases, cancer, diarrhea, skin infections, diabetes mellitus, hemorrhoids and other bacterial and fungal infections (Hajifattahi *et al.*, 2016). Gallic acid, punicallins and ellagic acid extracted from the pomegranates peel revealed good inhibitory effect against pathogenic microorganisms including ESBL-producing *Enterobacteriaceae* (Opara *et al.*, 2009). *Spondias dulcis* (SD) is a fast-growing equatorial tree with edible fruits which is popular in India in the name of Amra (Hog palm or golden apple). Though it is most commonly used as food source, the astringent bark is used as a remedy for diarrhoea in Cambodia (Morton, 1987). SD is also used in eyesight enhancement and eye infections (Rahmatullah *et al.*, 2009) and the fruit is used to cure itchiness, internal ulceration, sore throat and inflammation of skin. *Solanum torvum* (ST, hat begoon in Bengali) and parts of the plants are used as sedative, diuretic and digestive. They are also used in the treatment of coughs and colds. *Bryophyllum pinnatum* (Patharkuchi, BP) is a perennial succulent herb which grows in Africa and Asia (Siddiqui *et al*., 1989). Leaves or the whole plant are else where for treatment of ear infections, cough and dysentery (Akinpelu, 2000). Previous phytochemical studies revealed the presence of terpenoids and antileishmanial flavonoids in this plant (Siddiqui *et al*., 1989; Muzitano *et al.*, 2006). In addition, 60% methanolic extract of leaves of *B. pinnatum* have shown antimicrobial activity (Akinpelu, 2000).

The locally available herbs of ethno-medical importance have been traditionally used in treatment of several disorders of human was evaluated for their use in animal production as an alternative to AGP. Therefore, the study was designed to evaluate the *in-vitro* antibacterial effects of aqueous-methanolic extract of six herbs against ESBL-producing *E. coli* and *Salmonella* isolated from poultry.

MATERIALS AND METHODS

Preparation of plant extract: In this study, we attempted to evaluate the antimicrobial efficacy of aqueous-methanolic extract of six locally available ethano medical herbs viz. *Ocimum sanctum* Linn. (Tulsi) leaf, *Nyctanthes arbor-*

tristis (Seuli) leaf, *Solanum torvum* (wild eggplant) leaf, *Bryophyllum pinnatum* (Patharkuchi) and *Spondia dulchis* (*Amra*) fruit and *Punica granatum* (Pomegranate) peel against ESBL-producing *E. coli* and S*almonella* spp. isolated from poultry in west Bengal.

The collected samples included dry plant parts including leaves, fruits and peels. The aqueous methanol extracts were prepared from dry powder. The fresh samples were washed with distilled water, surface sterilized by 70% ethanol, and bleached with 5% aqueous sodium hypochlorite. The samples were again washed with double distilled water to make the samples chemical free. Thereafter, the samples were air dried at room temperature and finally crushed to powder in a grinder/mixer. The powdered samples were stored at -20°C till further use. The dry extracts were prepared using water and methanol (70%) as solvents. Ten percent aqueous and methanolic extracts were prepared as per the method of Ahmad and Beg (2001). Ten grams of powdered samples were soaked in 100 mL of solvent and kept for 24 hrs under continuous shaking, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatants were filtered by Whatman filter paper No. 1, dried by Rotary evaporator and lyophilized to obtain crude extracts and stored at -20°C.

Detection of antibacterial property of plant extracts: Antibacterial properties of the selected plant extracts were detected by agar well diffusion Method. Mueller-Hinton agar (HiMedia, India) was prepared, poured in Petri plates and allowed to solidify. One hundred microlitre of the selected ESBL producing *E. coli / Salmonella* cultures isolated from poultry (obtained from Department of Veterinary Microbiology, WBUAFS) possessing properties of both phenotypical and genotypical antimicrobial resistance were spread into the plates. The wells were made by well borer, 100 µL diluted sample was put in each well, and incubated overnight at appropriate temperatures (37°C). The bottom of the wells was sealed with agar to prevent the seepage of the agar. We checked antibacterial activity of aqueous/ methanolic extracts (50 mg/mL) of *Nyctanthes arbor-tristis* (NA, Seuli) leaf, *Ocimum sanctum* Linn. (OS, *Tulsi*) leaf, Punica granatum (Pomegranate, PG) pee, *Spondias dulcis* (SD, Amra) fruit, *Solanum torvum* (ST, wild eggplant) leaf and *Bryophyllum pinnatumn* (BP, Patharkuchi) leaf against *E. coli / Salmonella*. Zone of inhibition (ZOI) was measured to access the results. Gentamicin (10 mcg) was used as positive control as our recent study found gentamicin sensitive against *Enterobacteriaceae* isolates from local poultry birds (Chowdhury *et al.*, 2022).

Determination of minimum inhibitory concentration (MIC) of plant extracts against ESBL-producing E. coli isolated from poultry: MIC of plant extracts was detected by tube dilution method (Das *et al.*, 2016). In brief, 10 µL of bacterial strains in nutrient broth (0.5 McFarlands standard) were considered for the study. Different concentrations (1, 2, 5, 10, 25, 50, 100, and 200 µg/mL) of plant extracts were added to the test tubes containing the test strains. The positive control with gentamicin (50 µg/mL) was used with the test bacterial strains. After 24 hours of incubation in shaking condition, the MIC values were obtained by checking the turbidity of the bacterial growth. The lowest concentration at which there was no visible turbidity was considered as the MIC of that plant extract.

RESULTS

Detection of antibacterial property of plant extracts: The antibacterial activity of methanolic extracts of six selected plant extracts against ESBL-producing *E. coli* and *Salmonella* spp. from poultry is summarized in Table 1. The results revealed that all the six tested plant extracts showed antibacterial activity with varying magnitudes. *Ocimum sanctum* Linn. (Tulsi, 50 mg/mL), *Nyctanthes arbor-tristis* (Seuli, 50 mg/mL) and *Punica granarum* (Pomegranate, 50 mg/mL) were found most effective against ESBL-producing *E. coli* and

Table 1. Detection of antibacterial properties of plant extracts against ESBL- producing *E. coli* and *Salmonella*

Bacteria	NA (50 mg/mL)	OS (50 mg/mL)	PG (50 mg/mL)	SD (50 mg/mL)	ST (50 mg/mL)	BP (50 mg/mL)	Gentamicin (10 mcg)
ESBL-producing *Escherichia coli* (n=1)	13±1	15±1	14±1	11±1	10±1	14±1	12±1
ESBL-producing *Salmonella* (n=1)	13±1	14±1	13±1	11±1	10±1	14±1	12±1

Table 2. Minimum inhibitory concentration (MIC) of plant extracts against ESBL-producing *E. coli* isolated from poultry

Plant Extracts	MIC
Nyctanthes arbor-tristis	0.87 mg/mL
Ocimum sanctum Linn.	0.56 mg/mL
Punica granatum	0.64 mg/mL
Spondia dulchis	1.06 mg/mL
Solanum torvum	1.20 mg/mL
Bryophyllum pinnatum	0.98 mg/mL

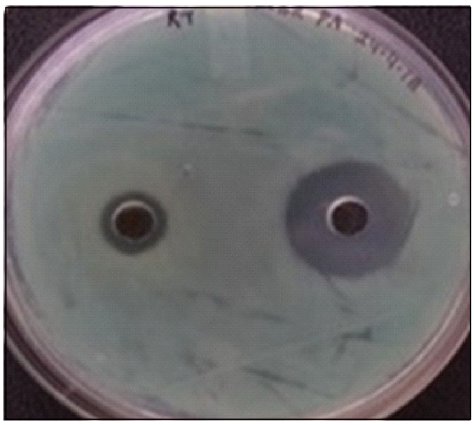

Fig. 1. Zone of inhibition produced by *Ocimum sanctum* Linn. (large zone) and *Spondia dulchis* (small zone) extracts against ESBL-producing *E. coli*

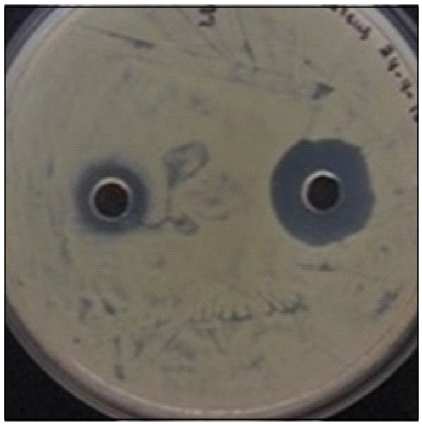

Fig. 2. Zone of inhibition produced by *Nyctanthes arbor-tristis* (large zone) and *Solanumtorvum* (small zone) extracts against ESBL-producing *E. coli*

Salmonella, which produced higher ZOI than the control antibiotic (gentamicin) (Fig. 1 and Fig. 2).

Determination of minimum inhibitory concentration (MIC) of plant extracts against ESBL-producing *E. coli* isolated from poultry: The minimum inhibitory concentration (MIC) of six selected plant extracts against ESBL-producing *E. coli and Salmonella* spp. from poultry is summarized in Table 2. The results showed that the MIC of the plant extracts

ranged from 0.56 mg/mL to 1.20 mg/mL. OS leaf extract produced lowest MIC value (0.56 mg/mL) against ESBL-producing *E. coli* and *Salmonella* among the studied plant extracts.

DISCUSSION

In this study, we attempted to evaluate the antimicrobial efficacy of aqueous-methanolic extract of six locally available ethano medical herbs viz. *Ocimum sanctum* Linn. (Tulsi) leaf, *Nyctanthes arbor-tristis* (Seuili) leaf, *Solanum torvum* (wild eggplant) leaf, *Bryophyllum pinnatum* (Patharkuchi) and *Spondia dulchis* (Amra) fruit and *Punica granatum* (Pomegranate) peel against ESBL-producing *E. coli* and *salmonella* spp. isolated from poultry in west Bengal.

Among a wide range of medicinal herbs, *Ocimum sanctum* Linn. stands out as one of the most investigated herbal medicine. OS leaves have antibacterial components such as eugenol, caryophyllene, germarene-A, clemene and caryophylline oxide mainly in the form of essential oils (Prakash and Gupta, 2005). OS leaf extract showed better antibacterial activity than the gentamicin and also from the rest of the tested extracts (Table 1, Fig. 1 and Fig. 2). OS leaf extract (50 mg/mL) had a lager ZOI against resistant *E. coli* and *Salmonella* spp. compared with that of gentamicin. However, few literatures are available for effects of aqueous-methanolic extract on *E. coli* and *Salmonella* spp. from poultry. Moreover, several studies revealed the efficacy of OS leaf EO and extract on both pathogenic Gram positive and Gram-negative bacteria from human origin (Mishra and Mishra, 2011). They reported that aqueous extract, alcoholic extract, chloroform extract and EO from OS leaf were equally effective against both Gram-positive and Gram-negative bacteria including *E. coli* and *Salmonella*. The EOs and biologically active compounds in fresh leaves of OS are effective against *E. coli*, *Shigella*, *Salmonella t*yphi, *Bacillus anthracis* and *Pseudomonas aeruginosa* (Prakash and Gupta, 2005). The extract of this plant was eugenol which was reported to have antibacterial, insecticidal and nematicidal activities (Chaterjee *et al*., 1982; Lemos *et al*., 2005).

Current study observed that NA leaf extract (50 mg/mL) had antibacterial effects with larger ZOI in comparison to the control antibiotics on ESBL-producing *E. coli* and *Salmonella.* Effects of aqueous-methanolic extracts of NA leaf on *E. coli* and *Salmonella* of poultry origin have not been studied so far. However, Priya and Ganjewala (2007) noted the antibacterial effects of ethyl acetate extracts (0.3 mL) on *E. coli* (19 mm of ZOI) in comparison to streptomycin (16 mm of ZOI) by disc diffusion method. ZOI in the present study differed from their finding, which may be due to variation in extraction method and concentration of extracts. Methanol was used in the present study, whereas DMSO was used as a solvent in the study conducted by Priya and Ganjewala (2007). Samuel *et al.* (2010) reported the antibacterial effects of methanolic NA seed extract with larger ZOI against *E. coli* (14 mm) and *Salmonella typhi* (15mm) at 600 mg/mL concentration. Thus, the antibacterial activity of NA leaf extract in this study agrees with previous works on *E. coli and Salmonella*. Jain and Singh (2013) reported the antibacterial effects of crude methanolic extract (0.04 mL/disc) against *E. coli* and *S. typhimurium* with ZOI, 23 mm and 22 mm respectively in comparison to antibiotic streptomycin (20 mm for *E. coli* and 19 mm for *Salmonella*). The antibacterial activities of plant parts were due to the presence of various plant secondary metabolites *viz*., glycosides and phenolics (Priya and Ganjewala, 2007). Phytochemical analysis of leaf, fruit and seeds of *N. arbor-tristis* revealed the presence of phytosterols, phenolics, tannins, flavonoids, glycosides and saponins. The secondary metabolites present in *N. arbor-tristis* are known to be biologically active and may act as antibacterial (Priya and Ganjewala, 2007). The antibacterial activities are attributed to

tannins which have been found to form irreversible complexes with proline-rich proteins, resulting in the inhibition of the bacterial cell protein synthesis. Flavonoids are phenolic structures containing one carbonyl group complexes with extracellular and soluble protein and with bacterial cell wall. Thus, it exhibits antibacterial activity (Cowan, 1999).

The present study reported antibacterial effects of aqueous-methanolic PG peel extract (50 mg/mL) against ESBL-producing *E. coli* and *Salmonella* with a larger ZOI compared with the gentamicin. However, the study has been done to evaluate the antibacterial effects of aqueous-methanolic PG peel extract on *E. coli* and *Salmonella* from poultry. The results of the present study are in agreement with Dey *et al.* (2012), where they reported the antibacterial effects of PG pericarp extract (1 mg/mL) on ESBL-*E. coli* with ZOI of 15 mm. They noted a similar MIC against tested *E. coli*. The antimicrobial potency of PG peel could be attributed to a high content of hydrolyzable polyphenols, mainly consisting of gallotannins and ellagitannins, such as punicalagins, punicalins and ellagic acid (Cowan, 1999; Reddy *et al.* 2007). In general, the inhibitory mechanisms of polyphenolic compounds are believed to be associated with the inhibition of microbial enzymes through reaction with sulfhydryl groups or by nonspecific interactions with the proteins (Cowan, 1999). Further, many plant-derived flavonoids have also been observed to act as anti-infective agents by virtue of their ability to form complexes with extracellular and soluble proteins in the bacterial cell walls (Cowan, 1999).

The current study revealed a moderate antimicrobial activity of methanolic fruit extract of *S. dulcis* against ESBL-producing *E. coli* and *Salmonella* with comparatively lesser ZOI than gentamicin. Result of this study is corroborative with Islam *et al.* (2013). The methanolic fruit extract of SD exhibited the highest phenolic content, flavonoid content and antioxidant capacity with the highest DPPH radical scavenging activity (Islam *et al.* 2013). Since plants are rich in various types of secondary metabolites including tannins, terpenoids, alkaloids and flavonoid, they have been found to exert *in vitro* antimicrobial property (Stanojevic *et al.*, 2009).

The present study revealed that aqueous-methanolic leaf extract of ST are not effective against ESBP-producing *E. coli* and *Salmonella.* ZOI produced by them are lesser than that of gentamicin. Similar result was obtained by Anatole *et al.* (2019), where they reported the inability of aqueous extract of ST leaf on multi-drug resistant *E. coli* and *Salmonella*. In other study, Melila *et al.* (2021) stated that hydro-ethanolic extract of ST fruit did not show antibacterial effects against *E. coli* and *Salmonella*. Extract of the fruits and leaves are said to be useful in case of hepatomegaly, splenomegaly, cough and cracks in feet (Belboukhari and Cheriti, 2006). Due to the notable medicinal value of ST, it was considered of interest to carry out a phytochemical and antimicrobial investigation of this species and the results leading to the antimicrobial screening are presented in this paper (Belboukhari and Cheriti, 2006).

Present study revealed the antibacterial effects of methanolic extract (50 mg/mL) of *B. pinnatum* (BP) leaf against ESBL-producing *E. coli* and *Salmonella* with larger ZOI compared to gentamicin. This result is similar to the work of Akinpelu (2000) and Ofokansi *et al.* (2005) that showed strong activities of methanol extract of BP against some Gram-positive organisms. The antimicrobial effect of methanol extract of BP leaf against these organisms may be due to the ability of the methanol to extract some of the active properties of these plants like phenolic compounds, saponin, bryophyllin and other secondary metabolites which are reported to be antimicrobial (Cowan, 1999; Okwu and Josiah, 2006).

The rationale behind the use of BP leaf extract in microbial infections and wound healing like skin infections, wound infection,

abscess and gastrointestinal disorder and ear infection in traditional medicine (Gill, 1992). Aibinu *et al*. (2003) reported that moderate antibacterial effects of methanol extract of BP leaf on *E. coli* and *Salmonella* with lesser ZOI than ciprofloxacin antibiotic. The present study also depicted a comparatively higher MIC than OS, PG and NA extract, although the ZOI was greater than them. Thus, ZOI may not be accurate measure of antibacterial effects, as ZOI depends on some other physical properties of the plant extracts. Thus, among all the tested plant extracts OS leaf extract, PG peel extract and NA leaf extract had better antibacterial effects than other tested plant extracts against ESBL-producing *E. coli* and *Salmonella*. Antibacterial effects of the aqueous-methanolic extracts may be due to the detrimental effects of the phytochemicals as phenolic compounds, alkaloids and flavonoids on the bacteria.

From the present study, it may be concluded that among the tested plant extracts aqueous/ methanolic extracts of all the plant extracts exhibit antibacterial effects against ESBL-producing *E. coli* and *Salmonella* from poultry. However, *O. sanctum* (Linn.) leaf extract, *P. granatum* peel extract and *N. arbor-tristis* leaf extract possess antibacterial effects with lower MIC, which may be used as a feed additive in poultry diet as an alternative to AGP.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: PK, AP and SM: Conducted the study; GPM and IS: Planned the study; IS: Received the grant and wrote the manuscript.

ACKNOWLEDGEMENT

The authors provide sincere thanks to the Honourable Vice Chancellor, West Bengal University of Animal and Fishery Sciences for providing the infrastructure and facilities to perform the research. The work was supported by the Department of Science & Technology and Biotechnology, Government of West Bengal (grant no: 1G-25/2016).

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Received – 28.03.2022, Accepted – 20.7.2022, Published – 04.08.2022 (Online), 01.12.2022 (Print)
Section Editor: Prof. S. N. Joardar, Associate Editor