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In vivo assessment of antibacterial and growth promoting effect of soursop (*Annona muricata* Linn.) leaf extract in broiler chickens

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Abstract

Phytochemicals have demonstrated considerable influence against pathogenic microbes *in vitro* without antimicrobial resistance. A number of studies have also reported an improved growth performance in poultry with their supplementation. *In vivo* antimicrobial efficacy of soursop leaf extract (SLE) was evaluated in a 49-day study on the growth performance (GP), intestinal microbes and morphology of broiler chickens. Two hundred day-old chicks (Arbor Acres) were randomly distributed to five treatments and four replicates, with ten birds each in a completely randomized design. The treatments included: CTRL (control), 5.0, 7.5, 10.0 and 12.5% SLE per litre of drinking water. Ciprofloxacin 10% was administered to the control group as antibiotics in drinking water at 1ml/2L. Feed intake (FI), weight gain (WG) and feed conversion ratio (FCR) were evaluated. Two birds per replicate were sacrificed on day 49 and eviscerated. Ileum and caeca content were collected and cultured for total coliforms, Lactobacillus, Salmonella and *Escherichia coli*. Mid-ileum section was cut, rinsed with distilled water and fixed in 10% formalin for evaluation of villus height and crypt depth. Analysis of variance was used for data analysis at $p<0.05$ confidence. Weight gain increased from 7.5% SLE while FI reduced from 10.0% and above giving the best FCR at 12.5% SLE. SLE improved WG, eliminated salmonella and reduced total coliform (1.30 to 0.00) in the ileum section. Total coliforms were eliminated, Salmonella and *Escherichia colli* decreased (6.02 to 1.85; 6.91 to 2.24) in the caeca section. It was concluded that SLE reduced pathogenic intestinal microbes, improved intestinal morphology and feed conversion ratio in broiler chickens.

Key Words: Growth Performance, intestinal microbes, Pytochemicals, soursop, leaf extract
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Introduction

Intestinal health is key to improved growth performance of birds and influences the efficiency of poultry production. Poor intestinal health results in delayed growth and high mortality in broiler chickens (Katouli, 2010). Plants such as neem, garlic, ginger, oregano, thyme, pawpaw, moringa etc, possess antimicrobial; antifungal; antioxidant, anti-inflammatory and antiparasitic effects and have been used as alternative growth promoters to enhance poultry production (Usunobun et al., 2015; Dhama et al., 2015). Phytochemicals such as alkaloids, flavonoids, glycosides and saponins can exert diverse effects including improved digestion, feed efficiency, reduced nitrogen excretion, improved gut flora and health status of animals (Kroismayr et al., 2008). Antibacterial effects of some herbs and plant extracts have been reported on both gram positive and gram negative bacteria (Shoeib and Alkufeidy, 2014; Joshi et al., 2020).

Another medicinal plant that is worth considering in poultry production is *Annona muricata*, commonly known as soursop. *Annona muricata* is a tropical fruit bearing tree used for several purposes. Soursop is native to Mexico, Cuba,

Central America and some parts of India. Soursop is widely used in traditional medicines in countries like Peru, Brazil and Togo where the extract is employed to treat diseases such as liver disorder, diarrhea, dysentery, fevers, hypertension, sores, internal ulcers and diabetes (Coria-Tellez et al., 2016). The leaves are reported to have multipurpose medicinal properties such as sedative, cytotoxic, anti-leishmanial, wound healing, antimicrobial, anti-carcinogenic and immune-boosting activities (Moghdamtousi et al., 2015). The leaves contain flavonoids, alkaloids, tannins, saponins, ascorbic acid, beta-carotenes, minerals and other nutrients (Usunobun et al., 2015).

Few studies have been reported on the positive effect of soursop extracts on microorganisms such as *Staphylococcus aureus*, *Vibrio cholera*, *Escherichia coli* and *Salmonella spp* (Vieira et al., 2010, Nasution et al., 2020). The potency of soursop leaf extracts against microorganism is based on the presence of acetogenins, alkaloids and phenols which are known to have tumoricidal, antimalaria, anthelmintic, antiparasitic, anti-inflammatory, antioxidant and antimicrobial effects, suggesting many potentially useful applications (Ferreira et al., 2013; Moghadamtousi et al., 2015; Mutakin et al., 2022). However, there is paucity of information on the antibacterial activity of soursop leaf extract in poultry production. Therefore, this study sought to assess the antibacterial and growth promoting potential of soursop leaf extract in broiler chickens.

Materials and Methods

Experimental site

The experiment was conducted at the poultry unit of Teaching and Research Farm, Federal College of Agriculture Ishiagu, Ebonyi State, Nigeria, situated at Latitude 5° 48' North of the equator and longitude 7° 31' East with an average rainfall of 1655 mm, prevailing temperature of 28.5°C and average relative humidity of 85%.

Source and processing of experimental materials

Fresh soursop leaves were harvested within Ishiagu, weighed (50, 75, 100, 125g), crushed with pestle in a mortar; a litre of water was added to the paste and filtered to obtain the desired soursop leaf extract (5%, 7.5%, 10% and 12.5%) concentrations for the experimental treatments.

Experimental Birds and Management

The experimental procedures were planned and performed in accordance with the guidelines of Animal Use and Care Committee (nvriAUCC F001/15) Vom, Nigeria. A total of 200 day-old unsexed Arbor acres broiler chicks were randomly assigned to five treatment groups, replicated four times with ten chicks each in a completely randomized design. The chicks were managed on deep litter with wood shaving as litter material in pen units of 4.0 x 4.0 ft. The experiment lasted for 49 days in two feeding phases (Table 1). The treatment groups were: CTRL (control), 5.0%, 7.5%, 10.0% and 12.5% leaf extract. The various percentages of soursop leaf extracts were prepared by crushing 50g, 75g, 100g and 125g of leaves in a mortar and extracting in a litre of water each. The extracts were administered to the birds for drinking. The control group received synthetic antibiotics (ciprofloxacin 10%*)* in drinking water at 1mL/2L without the leaf extract whereas treatments two to five received the leaf extract as prepared above without further dilutions. Both the extract and ciprofloxacin were administered throughout week one, week four and week six of the experiment. All the chicks were vaccinated against Newcastle and infectious bursal diseases and treated against coccidiosis.

Table 1: Percentage composition of experimental diet g/100g DM

Ingredients	Starter Diet (%)	Finisher Diet (%)
Maize	53.00	58.00
Groundnut cake	19.00	16.00
Soyabean meal	19.50	16.00
Palm kernel cake	4.00	6.00
Fish meal	1.00	1.00
Limestone	1.00	1.00
Bone meal	1.50	1.00
Salt	0.25	0.25
Vitamin/mineral premix	0.25	0.25
Methionine	0.25	0.25

Lysine	0.25	0.25
Total	**100**	**100**
Calculated Values		
Crude Protein (%)	23.22	21.13
Metabolizable Energy (Kcal/kg)	2961.00	3002.00
Calcium (%)	1.10	1.00
Phosphorus (%)	0.87	0.75
Methionine (%)	0.56	0.55
Lysine (%)	1.00	1.00
Crude fibre (%)	3.22	3.51

Sample Collection

Growth Performance indices (feed intake, weight gain and feed conversion ratio) were evaluated. Two birds per replicate were selected and sacrificed on day 49. The birds were dissected for the small intestine which were then separated into duodenum (the loop), jejunum (from the loop to meckel's diverticum), ileum (Meckel's diverticum to ileo-caecal junction) and caeca (branched blind pouches located where the small and large intestines joins). Digesta samples were collected from ileum (mid-point between Meckel's diverticum and ileocaecal junction) and caeca sections of the intestine into sterile bottles with lids and transported in ice box to the laboratory for microbial enumeration of Total coliforms, Lactobacilli, Salmonella, and *Escherichia coli*. One gram (1g) of digesta samples were serially diluted in sterile distilled water and aliquots of the dilutions were aseptically plated unto growth media; MRS (de Man Rogosa and Sharp) Agar was used to enumerate total lactobacillus species. The plates were anaerobically incubated at 37ºC for 3days. *Escherichia coli* was enumerated after incubation on MacConkey agar at 37°C for 48 h. The presence of pinkish to red colonies with a metallic sheen indicated *Escherichia coli* and colonies with a clear appearance on MRS agar indicated *Lactobacillus* species.

Salmonella-Shigella Agar was used to enumerate the total Salmonella population. The plates were incubated aerobically at 30ºC for 24 hours; the presence of Salmonella species was indicated by black colonies (Pepper and Gerba, 2005; Ofongo and Ohimain, 2019). After the incubation, colonies were counted and expressed as colony forming units (cfu)/g of the samples, bacterial counts were expressed as Log10 cfu.

Midsections of the ileum (3cm) were cut, flushed with distilled water and fixed in 10% formalin for histological evaluation. The tissue specimens were dehydrated with graded alcohol ranging from 70% to 100% in a sequential order and embedded in paraffin wax. The tissues were sliced using microtome into thin sections (2-3µm) which were placed in water bath for even spreading and were later picked up with glass slides and dried. After removal of paraffin wax, the slides were stained with hematoxylin and Eosin which impacts a red and navy blue colour to the tissues. The tissues were then mounted with a mountant (DPX) and covered with a thin glass cover-slip and allowed to dry. Using Leica DM 750M, the villus length was measured at X10 objective lens. A minimum of fifteen (15) villi were measured per sample and the average length were calculated. The microscopic measurements were re-converted to expected actual length by dividing with a factor of the objective lens magnification. Villus height was carried out on replicate basis with regards to each treatment (Ofongo and Ohimain, 2019).

Data Analysis

Data collected was subjected to one-way analysis of variance in a gross linear model of SPSS version 20. Duncan's multiple range test was used to separate the treatment means and the level of significance was taken at $p < 0.05$.

Results

The results of soursop leaf extract on growth performance of broiler chickens are presented in

Table 2, while that of intestinal morphology and microbes are presented in Tables 3 and 4.

Growth performance

Significant changes were observed in growth performance as a result of soursop leaf extract administration. The addition of 5% leaf extract depressed ($p<0.05$) feed intake and average daily weight gain leading to a poor feed conversion ratio as compared to the control and other treatments. However, the trend changed significantly with the administration of 7.5% leaf extract, which elevated feed intake to the peak with a corresponding high FCR. Feed intake thereafter decreased ($p<0.05$) at 10% and 12.5% SLE administration with corresponding better feed conversion ratios. The effect of soursop leaf extract on growth performance was observed to be dependent on the concentration of the leaf extract.

Table 2: Effect of soursop leaf extract on growth performance of broiler chickens

Parameters	**CTRL**	**5 % SLE**	**7.5 % SLE**	**10 % SLE**	**12.5 % SLE**	**SEM**
Initial weight (g)	362.50	362.50	362.50	362.50	362.50	-
Final weight (g)	4062.50^{a}	3460.00^{c}	4058.75^{a}	3887.50^{b}	3757.50^{b}	68.51
Feed intake (g)	8655.00^{ab}	8400.00^{b}	9282.50^{a}	8130.00^{b}	7347.50^{c}	198.63
Average feed intake (g)	176.63^{ab}	171.43^{b}	189.44^{a}	165.91^{b}	149.95^{c}	3.55
Weight gain (g)	3700.00^{a}	3097.50^{c}	3696.25^{a}	3525.00^{b}	3395.00^{b}	68.51
Average weight gain (g)	75.51^{a}	63.21^{c}	75.43^{a}	71.94^{b}	69.29^{b}	1.22
Feed conversion ratio	2.34^{b}	2.81^{a}	2.51^{ab}	2.31^{b}	2.16^{c}	0.76

Note: $^{a\,b\,c}$ means with different superscript within a row are significantly different ($p<0.05$).
CTRL: Control; SLE: Soursop leaf extract; SEM = standard error of mean

Intestinal morphology

Results of the intestinal morphology showed significant differences among the treatments on villus height and crypt depth. The highest villus height (1947.76 µm) was observed at 7.5% SLE, which was significantly higher ($p<0.05$) than all the other treatments and 10% SLE recorded the lowest value (734.61 µm). Similarly, the crypt depth was highest (556.12 µm) at 7.5% SLE but lowest (394.35 µm) in 5% SLE while the others were similar (Table 3).

Table 3: Effect of Soursop leaf extract on intestinal morphology of broiler chickens

Parameters	**CRTL**	**5 % SLE**	**7.5 % SLE**	**10 % SLE**	**12.5 % SLE**	**SEM**
Villus height (µm)	1173.27^{b}	1363.03^{b}	1947.76^{a}	734.61^{c}	1402.02^{b}	135.45
Crypt depth (µm)	450.25^{ab}	394.35^{b}	556.12^{a}	443.79^{ab}	432.18^{ab}	21.77

Note: $^{a\,b\,c}$ means with different superscripts within a row are significantly different ($p<0.05$).
CTRL: Control; SLE: Soursop leaf extract; SEM = standard error of mean

Intestinal Microbes

Ileum

The ileal microbial population counted in terms of total coliform, *E. coli*, Salmonella and Lactobacillus showed significant differences (p>0.05) among treatment groups (Table 4). Total coliform count significantly reduced from 1.30 in treatment one (control) to 1.04 in treatment three (7.5%). Meanwhile, no count was observed in treatment four (10%) and treatment five (12.5%) leaf extract. No count was observed for *E. coli* in all the treatment groups, Salmonella was only observed in the control group with no count in the extract treated groups.

Caeca

Escherichia coli and Salmonella count significantly ($p<0.05$) decreased from 6.91 and 6.02 in treatment one to 2.24 and 1.88 in treatment five as the concentration of leaf extract increased respectively. Total coliform was only present in the control group while lactobacillus count was not significant ($p>0.05$) across all the treatments.

Table 4: Effect of soursop leaf extract on intestinal microbial count of broiler chickens

Ileum (log 10 CFU g^{-1})	**CTRL**	**5% SLE**	**7.5% SLE**	**10% SLE**	**12.5% SLE**	**SEM**
Total coliform	1.30[a]	1.14[b]	1.04[c]	0.00	0.00	0.15
Escherichia coli	0.00	0.00	0.00	0.00	0.00	0.00
Salmonella	1.24	0.00	0.00	0.00	0.00	0.13
Lactobacillus	4.13[a]	2.61[b]	1.82[c]	1.64[c]	1.15[c]	0.26
Caeca						
Total coliform	1.77	0.00	0.00	0.00	0.00	1.18
Escherichia coli	6.91[a]	5.23[b]	2.84[c]	2.54[d]	2.24[e]	0.48
Salmonella	6.02[a]	1.36[b]	1.07[c]	1.08[c]	1.88[c]	0.52
Lactobacillus	3.11	2.83	2.85	2.87	3.03	0.14

Note: [a b c], means with different superscripts within a row are significantly different ($p<0.05$).
SLE: soursop leaf extract, SEM = standard error of mean

Discussion

Antimicrobial agents are substances that kill microorganisms (bactericidal) or inhibit microbial growth (bacteriostatic). Antimicrobial agents disrupt microbial processes or structures that are different from those of the host. They may hamper cell wall synthesis of the pathogen, inhibit microbial protein and nucleic acid synthesis, disrupt microbial membrane structure and function, or block metabolic pathways through inhibition of key enzymes (Ullah and Ali, 2017). The action of medicinal plants on microorganisms have been found to be related to the presence of certain substances such as alkaloids, glycosides, essential oils, gums, tannins, steroids, saponins, phlobatannins, flavonoids and a host of other compounds that are present in them (Sofowora, 1993; Oyagade et al, 1999).

Antimicrobial and growth promoting activity of soursop leaf extract at concentrations of 5.0%, 7.5%, 10.0%, and 12.5% evaluated in the present study showed significant effects on the birds. Result of growth performance revealed increase in weight gain from treatment three (7.5%) upward, reduced feed intake as concentrations increased giving rise to a better feed conversion ratio at higher concentrations of the leaf extract. Soursop leaf extract improved utilization of feed consumed by the birds. Improved growth performance especially feed conversion ratio and weight gain in this study agrees with the findings of Kroismayr et al. (2008) who reported that plant active ingredients (phytochemicals) can aid digestion, improve feed efficiency, gut flora and health status of animals. The enhanced growth performance of broiler chickens in this study could be attributed to the synergistic effect of the bio-active components in SLE which possess antimicrobial, antioxidant, anti inflammatory, gastro-protective and digestive enzymes stimulation properties (Moghadamtousi et al., 2014; Coria-Tellez et al., 2016)

Villus height and crypt depth are important indicators of nutrient utilization, health status and performance of animals. Attia et al. (2017) noted

that longer villi and lower crypt depth are indicators of better nutrient absorption and less tissue turnover. Improved villus height increases surface area for improved nutrient transport and absorption leading to improved performance of birds (Rajput et al., 2013). In this study, oral administration of soursop leaf extract to broiler chicken resulted in an increased villus height and crypt depth in the ileum section, leading to improved growth performance. Setiawan et al. (2018) also reported longer villi height and deeper crypt depth in ileum with the incorporation of cashew leaf extract in broiler chickens. Comparably, the dietary supplementation of broiler chicken diets with propolis and bee pollen was found to increase villus height and crypt depth (Prakatur, 2019). This is however, contrary to the result of Rajput et al. (2013) who supplemented broiler diets with curcumin and observed longer villi with shorter crypt depth. Abdul et al. (2020) also reported longer villi with a shallow crypt on the supplementation of phytogenic feed additive in broiler chickens. The growth promoting effect of soursop leaf extract is demonstrated by improving intestinal development for better nutrient absorption.

Antibacterial effects of soursop leaf extract showed a significant decrease in total coliform in the ileal section at higher concentrations of the leaf extract. Salmonella was completely eradicated by the leaf extract at all concentrations while *E. coli was* not observed in all the treatments. In the caecal section, *E. coli* and salmonella count decreased with increased concentrations of the leaf extract while total coliform was totally eradicated by the extract. Lactobaccilus is one of the beneficial anaerobic bacteria that is mostly dominant in the caeca where fermentation of undigested food particles occur, its dominance in the chicken's intestine affords the chicken a healthy status and improved productive performance.

All concentrations of extracts were found to be effective against pathogenic microbes but 12.5% exhibited the highest antimicrobial effect on all microbes assessed except lactobacillus. The result in this study correspond to that of Vieira et al. (2010) who reported antimicrobial efficacy of soursop leaf extract against gram-negative and gram-positive organisms (*Staphylococcus aureus, Vibrio cholerae, Escherichia coli* and *Salmonella spp) in vitro*. Antibacterial effect of methanolic and aqueous extract of soursop leaves were also reported against *Proteus vulgaris*, *Streptococcus Pyogenes*, *Bacillus subtilis*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* (Abdusalami et al*.,* 2016). Another comparative study of the antibacterial activity of aqueous and ethanolic extracts of soursop leaves *in vitro* revealed that both extracts were effective against *Escherichia coli* and *Klebsiela pneumoniae* (Kamath et al., 2017). The antibacterial effect of soursop leaf extract is believed to be provided by the presence of saponins, tannins, flavonoids and alkaloids which act in diverse ways to either inhibit or destroy bacterial cells. Iyanda-Joel et al. (2019) also reported that extracts from different parts of soursop containing alkaloids showed antimicrobial activity against different multidrug resistant bacteria, namely *E. coli, P. aeruginosa, P. mirabilis, S. aureus, B. subtilis, Raoultella planticola, Enterobacter aerogenes, Agrobacterium tumefaciencs*, and pneumonia. The antimicrobial effect of the leaves is in no doubt a result of individual and synergistic effects of the phytochemicals present. It is clear that soursop leaf extract possesses potent broad-spectrum antibiotic activity, which validates its use for treatment of various microbial infections in traditional medicine. Its efficacy has been tried *in vitro* with success stories and *in vivo* trials in this study has justified its use as alternative antimicrobial and growth promoting agent in poultry. Soursop leaf extract exhibited higher growth promoting and antibacterial effect against the listed pathogenic microbes compared to the synthetic antibiotics used as control. Further research is required to establish the effective use of soursop leaf extract in maintaining intestinal health and promoting growth of broiler chickens.

Conclusion

Soursop leaf extract between the concentrations of 7.5 - 12.5% improved feed efficiency, intestinal morphology, promoted growth of broiler chicken and significantly reduced intestinal microbial count (total coliform, salmonella and *Escherichia coli*) in broiler chicken. Growth promoting and antimicrobial effect of soursop leaf extract was observed to increase with concentration, giving the best result for feed conversion and microbial count at 12.5% leaf extract while intestinal morphology was best at 7.5% SLE. This result provides the basis for utilization of soursop leaf extract as a natural antibacterial and growth promoter in broiler chickens production.

Conflict of Interest: The authors declare there is no conflict of interest.

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