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Activity of ethanolic extract of *Eucalyptus globulus* leaves against multi drug resistant poultry pathogens in broiler chicks

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Abstract: The present study was designed to evaluate the antimicrobial activity of *E. globulus* leaves in broiler chicks. Total (n=255) day-old chicks were segregated into five groups i.e. Pathogenic *E. coli*, *S. pullorum*, *S. gallinarum* and *C. perfringens* type *A* and control negative group. Each bacterial challenged (1x 10^7 CFU) group was divided into control positive, antibiotic, probiotic and *E. globulus* group. Experimental birds were exposed to *E. coli, S. pullorum, S. gallinarum* and *C. perfringens* type *A* at different ages. At 35th day of experiment the log reduction for each group was determined. The highest log reduction in *E. coli* and *C. perfringens* Type *A* colonies count were found in *E. globulus* (3.26) (2.33) treated group followed by antibiotic (2.85) (1.59) and probiotic (2.84) (1.50) respectively. The log reduction in *S. pullorum* colonies count was highest in *E. globulus* (2.50) followed by probiotic (2.24) and antibiotic (2.16). The *S. gallinarum* colonies count log reduction was found highest for antibiotic (2.84) followed by probiotic (2.48) and *E. globulus* group. The results of *in-vivo* experiment revealed that ethanolic extract of *E. globulus* has antibacterial activity and it can be used as a replacement to low level of antibiotics added in poultry feed.

Key words: *Eucalyptus globulus; Escherichia coli; Salmonella pullorum; Salmonella gallinarum; Clostridium perfringens* type A.

Introduction

Livestock plays a momentous role in the country economy by bequest 11.22 percent in national GDP and 60.54 percent in the agriculture sector. The poultry sector is one of the fastest-growing sectors in Pakistan. It plays a vital role in the provision of proteinaceous food and job opportunity to masses. Resultantly, it regulates the country's economy. The attached sector like feed, medicine, vaccination, and processed food has a contribution in public sector mobilization and protein providence to people. It provides jobs directly and indirectly to 1.5 million people (1). The current investment is 200 billion. Pakistan is 11th the largest poultry producing country with the production of 1.2 billion broilers annually. It is considered the backbone of the country because it is run through over 7 million metric tons of agriculture ingredients. Poultry meat comes through 30 percent of total meat produced in the country (2, 3). In the poultry industry, broiler farming has been advantageous to farmers in terms of better and quicker turnover due to the short rearing period and higher profit margin. The higher feed cost and disease outbreaks are the major obstacles in the propagation of poultry business in the country. Feed make up 70% of the total cost of production and least-cost ration formulation is the focus of nutritionists(4).

Heavy economic losses are inflicted by the country due to heterogeneous infectious diseases which mannered a weighty threat for the survival of poultry farming especially at small scale level(5, 6). For a long time, antibiotics are extensively used in poultry the sector as prophylactically to prevent infections or as growth promoters to improve growth and health. However, problems attached to the application of antibiotics as growth promoters (AGPs) in a meat-producing animal include cross-resistance, drug toxicity, and residual effects in humans (7, 8). Consequently, the negative impact of antibiotics has pushed EU (European Union) to ban their use as AGPs in animal production since 2006 and this led to important consequences in diet formulation which make feed manufacturing exponentially complex in terms of traceability and accountability of feeds and their ingredients, the consumer perceptions have more weight on the safety and quality of animal products(9). The radical poultry disease incorporate Newcastle disease, *E. coli* infection, infectious bronchitis, infectious coryza, coccidiosis, Enteritis, Salmonellosis, Hydro pericardium and avian influenza (10). The high use of antibiotics in poultry industry to increase production had led to bacterial resistance. Due to the rise in health issues, the developed countries have banned the use of antibiotics in poultry feed. (11).

The first two weeks of age is very important in the life of a broiler chick. Out of total mortality, 30–50 percent occur in this period(12). These pathogenic organisms are major causes of early broiler losses. These microbes cause economic devastation in billions, every year(13).

To achieve quick target weight and better FCR in less days, antibiotics are added in low level in poultry feed. Large scale prophylactic use of antibiotics in the poultry industry results in increased antibiotic resistance and antibiotic residues are directly transferred into the human food chain. The researchers are searching for alternatives to antibiotics. Phytobiotics are one of the best sources in available resources. Plants, herbs and vegetable are recommended as feed additive or growth promoters in broiler rations to enhance the growth, FCR, feed efficiency and reduce the production cost (14). *Eucalyptus globulus* leaf powder had a positive effect on growth performance and feed intake which was associated with the manipulation of gut microbiota and improved immunity (15).

E. globulus is an ancient medicinal plant. *E. globulus* composed of phenolic compounds includes 1, 8-eucalyptol, a-pinene, a-tepineol, globulol, flavonoids, tannins and hydroxybenzoic acids (16) . These have several biological activities, including anti-carcinogenic, cardio protective, anti-inflammatory, antibacterial and antiviral properties attributed mainly to their antiradical, antioxidant activity and enhance nutrition and animal health. Recent studies in veterinary medicine show that these effects are reflected in a better growth performance in different species of food producing animals (17). Tannin interfere with enzyme activities and to cause morphological damage to the gut (18) and these mechanisms appear to decrease feed intakeand nutrient absorption but have significant effective as antimicrobial and antioxidant (19). Therefore, the objectives of the studies were to evaluate the antibacterial activity of ethanolic extract of *E. globulus* in broiler birds.

Materials and Methods

Experimental design and shed preparation for *in-vivo* evaluation trial

Antibacterial properties of *E. globulus* against common poultry pathogens including groups; 1) Pathogenic *E. coli.* 2) *S. pullorum* 3) *S. gallinarum* 4) *C. perfringens* type *A* 5) Control negative

In-vivo evaluation experimental shed was prepared for placement of day-old broiler chicks. The shed was properly cleaned and disinfected. All chicks received were kept in brooding temperature with facilitation and circulation of fresh air into the shed. Commercially available feed was specially made for experimental birds and divided into two groups. One group was composed of antibiotic-free feed and another group antibiotic was added in feed having same energy and ingredients.

In-vivo evaluation of antimicrobial activity of ethanolic extract of *Eucalyptus globulus* leaves against *E. coli*, *S. pullorum*, *S. gallinarum* and *C. perfringens* type A in broiler chicks

Total (n=255) day-old chicks were segregated into five groups i.e. Pathogenic *E. coli* (n=60), *S. pullorum* (n=60), *S. gallinarum* (n=60) and *C. perfringens* type *A* (n=60) and control negative group (n=15). Each bacterial challenged (1x 10^7 CFU) group was divided into control positive, antibiotic, probiotic and *E. globulus* group (n=15 birds). Experimental birds were exposed to *E. coli, S. pullorum, S. gallinarum* and *C. perfringens* type *A* at age of day six, nine, 20 and 27 respectively. At 35^{th} day of experiment the log reduction for each group was determined.

Enumeration of bacteria in poultry droppings sample

Conventional microbiological techniques were used for the enumeration of bacteria in dropping samples. Samples were homogenized and serially decimal dilution (10^{-1} to 10^{-7}) was prepared in peptone water. Each dilution was spread on a plate with L shaped glass spreader on selective media for *E. coli*, *S. pullorum*, *S. gallinarum* and *C. perfringens* Type *A*. For the enumeration of *E. coli*, count EMB agar (Italy), SS agar for *S. pullorum*, *S. gallinarum* and RCM media was used for *C. perfringens* Type *A*. Results were expressed as $\log_{10}$ colony-forming units per gram of fecal contents (20).

Results and discussion

In-vivo evaluation trial of ethanolic extract of *Eucalyptus globulus* leaves

In-vivo evaluation trials of ethanolic extract of *E. globulus* showed antibacterial activity against common poultry pathogens including *E. coli* is given in table (1), *S. pullorum* is given in table (2), *S. gallinarum* is given in table (3) and *C. perfringens* type *A* is given in table (4). Antibacterial activity of *E. globulus* was demonstrated by exposure of experimental birds to *E. coli*, *S. pullorum*, *S. gallinarum* and *C. perfringens* type *A*. Log reduction of bacterial colonies in birds fecal was anticipated and analyzed among experimental groups. It was detected that there was a substantial down turn in colony count in *E. globulus* group as compare to the control group and other experimental groups at 35^{th} day of age. There are a set of researches on the *In-vitro* antibacterial activity of *E. globulus* and plant extracts as compare to *in-vivo* research studies. The reason behind and the recommendations were that *in-vivo* there may be the higher opportunity of nutrients in food or body for microbes, and they repair their damaged cell parts quickly as correlate to their production in culture media (21). Another view, mentioned that the significant level of fat and protein present in food, serve as a protective shield for bacteria against the action of plant extract (22).

Another researcher reported the antimicrobial effect of *E. globulus* in vitro. The minced beef meat was inoculated with *E. coli* and *S. aureus* and was the meat is stored at 2-5 c. It was found that there was bacterial growth occurred in the control group having log CFU/g was 8.20 and 8 for *E. coli* and *S. aureus* respectively. There was bacterial growth inhibition in *E. globulus* supplemented group. There was a significant difference found between the treated and untreated groups in colony count. The *E. globulus* supplemented group declined the log reduction of *S. aureus* to 2.50 log CFU/g. The results for the in-vitro inhibition of *E. coli* and *S. aureus* in infected meat were in agreement with our research studies and results. It was also found in the results that *E. globulus* has more antibacterial activity against gram-positive bacteria as compared to the gram-negative bacteria (23). Another

Table 1. Effect of *E. globulus* extract on control of *E. coli* in broiler as determined by *E. coli* count and its log reduction at different days of trial.

Groups	Enumeration (Mean ±SD log10) and log10 reduction of E. coli at different days of experimental trial																
	Day 5	Day 7		Day 8		Day 11		Day 13		Day 17		Day 21		Day 28		Day 35	
	Count	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD
Negative Control	5.865± 0.415	6.019± 0.210	-	6.285± 0.309	-	6.708± 0.663	-	5.889± 0.547	-	5.275± 0.382	-	5.398± 0.634	-	5.417± 0.594	-	5.537± 0.672	-
Positive control (Challenged)	5.649± 0.640^{a}	7.264± 0.629^{a}	-	7.653± 0.418^{b}	-	7.624± 0.570^{a}	-	7.820± 0.694^{b}	-	7.891± 0.490^{b}	-	8.715± 0.515^{b}	-	8.585± 0.645^{b}	-	8.589± 0.555^{b}	-
E. globulus	5.912± 0.285^{a}	7.187± 0.534^{a}	0.076	6.220± 0.645^{a}	1.429	6.014± 0.434^{b}	1.609	5.953± 0.462^{a}	1.866	5.428± 0.440^{a}	2.462	6.578± 0.364^{a}	2.136	5.103± 0.393^{a}	3.481	5.324± 0.409^{a}	3.264
Antibiotic	6.168± 0.206^{a}	6.730± 0.718^{a}	0.533	6.563± 0.703^{ab}	1.086	6.148± 0.509^{b}	1.475	6.221± 0.634^{a}	1.598	5.810± 0.299^{a}	2.08	6.237± 0.515^{a}	2.477	5.5311± 0.103^{a}	3.053	5.730± 0.461^{a}	2.858
Probiotic	6.324± 0.391^{a}	7.427± 0.437^{a}	-0.163	6.672± 0.608^{ab}	0.977	6.225± 0.552^{b}	1.398	6.294± 0.456^{a}	1.525	5.780± 0.333^{a}	2.11	6.159± 0.497^{a}	2.555	5.688± 0.449^{a}	2.89	5.740± 0.391^{a}	2.848

S.D-Standard deviation, LD-Log reduction, Probiotic-Galliprotect, Day 5-Before challenge, Day 6-challenge with E. coli.

Table 2. Effect of E. globulus extract on control of S. pullorum in broiler as determined by S. pullorum count and its log reduction at different days of trial.

Groups	Enumeration (Mean ±SD log10) and log10 reduction of S. pullorum at different days of Trial																
	7	9		11		13		15		19		27		31		35	
	Count	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD
Negative Control	5.865± 0.415	6.019± 0.210	-	6.285± 0.309	-	6.708± 0.663	-	5.889± 0.547	-	5.275± 0.382	-	5.398± 0.634	-	5.417± 0.594	-	5.537± 0.672	-
Positive control (Challenged)	5.649± 0.640^{a}	7.224± 0.639^{a}	-	7.259± 0.604^{a}	-	7.675± 0.501^{a}	-	8.228± 0.539^{a}	-	8.213± 0.577^{b}	-	9.187± 0.480^{b}	-	5.929± 0.499^{a}	-	8.485± 0.405^{b}	-
E. globulus	5.912± 0.285^{a}	8.271± 0.691^{ab}	-1.048	7.197± 0.432^{a}	0.062	7.175± 0.676^{a}	0.5	7.133± 0.514^{a}	1.094	6.332± 0.481^{a}	1.881	6.867± 0.644^{a}	2.319	6.204± 0.391^{a}	2.676	5.979± 0.601^{a}	2.51
Antibiotic	6.168± 0.206^{a}	8.781± 0.639^{b}	-1.558	7.707± 0.633^{a}	-0.448	7.309± 0.537^{a}	0.366	7.369± 0.641^{a}	0.858	6.834± 0.600^{a}	1.379	7.283± 0.489^{a}	1.903	6.186± 0.409^{a}	2.401	6.318± 0.536^{a}	2.17
Probiotic	6.324± 0.391^{a}	8.724± 0.728^{b}	-1.501	7.698± 0.656^{a}	-0.439	7.222± 0.471^{a}	0.453	7.346± 0.610^{a}	0.881	6.771± 0.562^{a}	1.442	7.304± 0.498^{a}	1.882	8.605± 0.494^{b}	2.419	6.240± 0.556^{a}	2.24

S.D-Standard deviation, LD-Log reduction, Probiotic-Galliprotect, Day 7-Before challenge, Day 8-challenged with *S. pullorum.*

author reported that methicillin-resistant *S. aureus* infection can be significantly declined by the application of *E. globulus* oils (24).

Antibacterial activity of ethanolic extract of *E. globulus* leaves against ***E. coli*, *S. pullorum, S. gallinarum*** **and *Clostridium perfringens* type *A***

The log reduction colony count method opted by (25) to find out the antimicrobial properties of *E. globulus in-vivo*. The result indicated that *E. globulus* had declined the bacterial growth and there was decline occurred in colony counting of *S. aureus*. The log reduction noted for *S. aureus* (25923 and 1) was 2.28 and 4.57. Similar microbial reduction appeared in water (26) using of *E. globulus* extracts. It was established that maximum microbial reduction 13% was occurred at pH 7. The microbial reduction decreases 11% and 10% when pH increases 7.05 and 7.1, when pH was 6.9 and 6.95 the microbial reduction was 12 and 11 percent. pH is one of the important biotic elements that work as an indicator for pollution.

E. coli exposure to four experimental birds' groups (Positive control, ethanolic extract of *E. globulus*, probiotic and antibiotic) on day 05 of its age revealed that there is no log reduction in bacterial colony count as compare to positive control which is mentioned in table (1), and no challenge was given at day 05. The birds were challenged at day 06, after day 06 the log reduction was measured which is given below in reference table (1). The highest log reduction was occurred at day 35th for all three groups. The most significant log reduction was noted in group feed with *E. globulus* which is 3.26, while probiotics and antibiotics have significant and same log reduction occurred at day 35 given in table (1).

Exposure of *S. pullorum* to four experimental bird groups (Positive control, ethanolic extract of *E. globulus*, probiotic and antibiotic) on day 07 of its age revealed that there is no log reduction in bacterial colony count as compare to positive control which is mentioned in table (2), and no challenge was given at day 07. The birds were challenged at day 09, after day 09 the log reduction was measured which is given below in reference table (2). The highest log reduction was occurred at day 35th for all three groups. The most significant log reduction was noted at day 35th in group feed with *E. globulus* extract, which were 2.51, followed by probiotic (2.24) and antibiotic (2.17) groups given in table (2).

Challenge of *S. gallinarum* was given to four experimental birds' groups (Positive control, ethanolic extract of *Eucalyptus globulus*, probiotic and antibiotic) on day 19 of its age revealed that there is no log reduction in bacterial colony count as compare to positive control which is mentioned in table (3), and no challenge was given at day 19. The birds were challenged at day 20, after day 20 the log reduction was measured which is given below in reference table (3). The log reduction was low at day 29 for *E. globulus,* probiotic and antibiotic group i.e. 0.99, 0.79 and 0.78 respectively. At day 31 the log reduction was highest for *E. globulus* which is 2.32, while antibiotic and probiotic group log reduction was quite similar that was 1.88 and 1.79 respectively. The highest log reduction was occurred at day 35 for all three groups. The most significant log reduction was noted at day 35 in group feed with antibiotics, which was 2.49, followed by antibiotic i.e. 2.41. Significant log reduction was also found in *E. globulus* group 2.24 at day 35 given in table (3).

C. perfringens type A exposure to four experimental birds groups (Positive control, ethanolic extract of *E. globulus*, probiotic and antibiotic) on day 26 of its age revealed that there is no log reduction in bacterial colony count as compare to positive control which is mentioned in table (4), and no challenge was given at day 26. The birds were challenged at day 27, after day 27 the log reduction was measured which is given below in reference table (4). The most significant log reduction was noted at day 35 in group feed with *E. globulus* extract which is 2.33, followed by antibiotic and probiotic group i.e.1.59 and 1.51 respectively given in the table (4)

In conclusion, the medicinal plant extracts are now considered the healthy and safest way to replace the antibiotics used in the poultry sector. *E. globulus* has antimicrobial activity and can be used as an alternative to antibiotics in poultry feed.

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Conflict of interest
There is no conflict of interest to declare.

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Table 3. Effect of *E. globulus* extract on control of *S. gallinarum* in broiler as determined by *S. gallinarum* count and its log reduction at different days of trial.

Groups	Enumeration (Mean ±SD log10) and log10 reduction of S. gallinarum at different days of Trial												
	19	21		23		25		29		31		35	
	Count	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD
Negative Control	5.865± 0.415	6.019± 0.210	-	6.285± 0.309	-	6.708± 0.663	-	5.889± 0.547	-	5.275± 0.382	-	5.398± 0.634	-
Positive control (Challenged)	5.544± 0.023^{b}	7.619± 0.219^{b}	-	7.241± 0.098^{c}	-	7.207± 0.087^{c}	-	6.876± 0.290^{b}	-	7.357± 0.133^{c}	-	8.218± 0.199^{b}	-
E. globulus	6.407± 0.038^{a}	6.709± 0.18^{a}	-0.09	6.006± 0.325^{a}	1.235	5.833± 0.408^{a}	1.374	5.877± 0.191^{a}	0.999	5.043± 0.214^{a}	2.32	5.977± 0.067^{a}	2.24
Antibiotic	6.770± 0.18^{b}	7.398± 0.325^{b}	-0.78	6.511± 0.408^{b}	0.73	6.109± 0.191^{b}	1.098	6.090± 0.214^{a}	0.786	5.482± 0.067^{b}	1.88	5.733± 0.182^{a}	2.48
Probiotic	6.755± 0.325^{b}	7.455± 0.408^{b}	-0.836	6.579± 0.191^{b}	0.662	6.170± 0.214^{b}	1.037	6.096± 0.067^{a}	0.78	5.560± 0.182^{b}	1.79	5.810± 0.163^{a}	2.41

S.D-Standard deviation, LD-Log reduction, Probiotic-Galliprotect, Day 19-Before challenge, Day 20-challenged with *S. gallinarum.*

Table 4. Effect of E. globulus extract on control of C. perfringens type A in broiler as determined by C. perfringens type A count and its log reduction at different days of trial.

Groups	Enumeration (Mean ±SD log10) and log10 reduction of C. perfringens type A at different days of Trial										
	26	28		29		31		33		35	
	Count	Count	LD	Count	LD	Count	LD	Count	LD	Count	LD
Negative Control	5.865± 0.415	6.019± 0.210	-	6.285± 0.309	-	6.708± 0.663	-	5.889± 0.547	-	5.275± 0.382	-
Positive control (Challenged)	5.521± 0.096^{a}	7.547± 0.047^{b}	-	6.867± 0.216^{b}	-	7.023± 0.110^{c}	-	6.987± 0.182^{c}	-	7.438± 0.116^{c}	-
E. globulus	5.652± 0.116^{a}	6.946± 0.011^{a}	0.6	5.915± 0.090^{a}	0.952	5.807± 0.046^{a}	1.216	5.656± 0.260^{a}	1.33	5.108± 0.060^{a}	2.33
Antibiotic	6.374± 0.141^{b}	7.501± 0.065^{b}	0.045	6.552± 0.087^{a}	0.315	6.073± 0.103^{b}	0.95	6.134± 0.099^{b}	0.85	5.839± 0.264^{b}	1.59
Probiotic	6.502± 0.093^{b}	7.569± 0.010^{b}	-0.023	6.608± 0.102^{a}	0.259	6.127± 0.100^{b}	0.896	6.191± 0.078^{b}	0.79	5.930± 0.283^{b}	1.51

S.D-Standard deviation, LD-Log reduction, Probiotic-Galliprotect, Day 26-Before challenge, Day 27-challenged with Clostridium perfringens type A.

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