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In vitro evaluation of antimicrobial and immunomodulatory efficacy of *Achyranthes aspera* extract in broiler chicks

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Abstract
Indiscriminate use of antibiotic have caused microbial resistance and also lead to many side effects to overcome from such situation plants and plant products are widely used for the treating of various ailments having antimicrobial and immunomodulatory properties. Therefore, present study, was undertaken to study *in vitro* antimicrobial and immunomodulatory activity of *Achyranthes aspera* extract against Pathogenic Microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella gallinarum* and *Pseudomonas aeruginosa*. Methanolic extract of aerial part of *Achyranthes aspera* were prepared and tested by "Disc Diffusion Method". Extract of *Achyranthes aspera* revealed antibacterial activity against both gram-positive bacteria (*S. aureus*) and gram-negative bacteria (*E. coli, Salmonella gallinarum and P. auriginosa*). For the study of cell mediated immunity 18 chicks were divided into 3 treatment groups (T1, T2 and T3) 6 chicks in each replicates. Similarly, for humoral immunity study, 18 chicks were divided into another 3 treatment groups of 6 chicks in each replicates. Each bird of different groups was individually identified by using leg band. T1 (Control diet), T2 (Standard growth promoter; BMD @ 0.05% in feed), T3 (*Achyranthus Aspera* powder @ 20g/kg feed of drinking water) for study of immunomodulatory effect for consecutive 42 days. Preliminary phytochemical screening of AAE revealed the presence of alkaloids, saponin, and phenol *Achyrantus aspera* extract showed significantly higher skin thickness in DNFB skin sensitization test both at 24 hours and 48 hours after sensitization, but did not show any significant effect on humoral immune response compared to the control group. In conclusion methanolic extract of *Achyranthus aspera* found to have potent antibacterial activity against various pathogenic organisms and immunomodulatory activity in broiler chicks.

Keywords: Antibacterial activity, *Achyranthes aspera extract*, (AAE), Disc diffusion method, Cell mediated immune, Humoral immune response, *Achyranthus aspera* powder (AAP)

1. Introduction
Folk medicine provides an important and unexplored resource for the discovery and development of potential new medicines against microbial infections to decrease the emergence of resistance and adverse drug reactions. Furthermore, the Plants are used in traditional and Ayurvedic systems of medicine as well as in tribal medicine in India and some other countries for primary health care because of their wide biological and medicinal activities, higher safety margin and lower costs (Ali *et al.* 2001) [1]. *Achyranthes aspera* Linn. (Family – Amaranthaceae) is commonly known as Latjira in Hindi. The plant is used by traditional healers for the treatment of dysentery, fever and diabetes (Sutar *et al.* 2011) [17]. Plant contain biologically active ingredients, for treating mild or chronic ailments. Alkaloids, tannins, flavonoids, and phenolic compounds are the most important bioactive constituents of this plant (Srivastav *et al.* 2011) [16]. It is described as bitter, pungent, purgative, laxative, stomachic, carminative and digestive and is useful for the treatment of bronchitis, heart maladies, piles, itching abdominal problems, ascites, rheumatism, abdominal enlargement, rabies and for enlarged cervical (Neeta *et al.* 2011; Dey, 2011; Dwivedi *et al.* 2008) [9, 3, 4]. It is active against cancer cells, used in dermal wound healing and improving gastro-protective activity, and in diabetes mellitus.

2. Materials and Methods
2.1 Plant materials
Achyranthes aspera (aerial parts) were collected from agricultural land and waste land from Durg district (C.G.) and was identified and authenticated by the Department of Botany Govt.

V.Y.T.P.G. Autonomous College, Durg (C.G.). Collected plant material was shed dried and powdered. The powdered plant material (*Achyranthes aspera*) was extracted using methanol in Soxhlet's apparatus for 16–24 hour or until the solvent was clears at 50-60 ºC. Extracts was concentrated using hot water bath. The extracts was kept in air tight screw cap vials, labeled and stored in refrigerator for use whenever required. This methanolic extract of *Achyranthes aspera* is referred here after as "*Achyranthes aspera* extract" (AAE). The dried extracts were then stored in an air tight jars at 4 ºC for antibacterial analysis.

2.2 Formulation of plant extract
The dried leaf powder of *Achyranthus Aspera* were extracted with methanol by using Soxhlet's apparatus at 50-60 °C for about 18 hours. About 50 gm of each powder was extracted with 250 ml methanol. The extracts were concentrated to dryness in hot water bath to yield crude residue. The extract was weighed and the percentage of yield was calculated. Obtained extract was stored in a sterile screw cap bottle and preserved in a refrigerator for further use.

2.3 Experimental animals
For immunological studies a clinically, healthy Thirty six (36) day- old broiler chicks were procured from Simran hatcheries, Raipur. For study of cellular immunity 18 chicks were divided into 3 treatment groups (T1 Control, T2-BMD @.05% and T3 *Achyranthus Aspera* powder 20g /Kg of feed 6 chicks in each. Similarly, for humoral immunity, 18 chicks were divided into another 3 treatment groups, 6 chicks in each group. Each bird of different groups was individually identified by using leg bands (Table 1).

Table 1: Experimental design for immunological studies in broiler birds

Particulars	Groups/ Treatments		
	Control	Standard	Test
	T_1	T_2	T_3
Cell mediated immunity study	6	6	6
Humoral immunity study	6	6	6
Basal feed	+	+	+
Bacitracin Methylene Disalicylate (% in Basal feed)	-	0.05	-
Achyranthus Aspera powder (AAP) feed	-	-	20g/Kg

2.4 Ethical consideration
The experiment was dully approved by the institutional Ethics Committee (IAEC) of the university (Permission no.445 IAEC/2016)

2.5 Micro-organism
Pure cultures of Pathogenic strains of *Escherichia coli, Staphylococcus aureus, Salmonella gallinarum* and *Pseudomonas aeruginosa* were obtained from the Post-Graduate Institute of Veterinary and Animal Sciences, Akola (MAFSU, Nagpur), where the bacterial cultures were originally obtained from Chandigarh (IMTch). They were sub-cultured on nutrient agar and in nutrient broth and maintained in the Department of Veterinary Microbiology, College of Veterinary Science & AH, Anjora, Durg and were used in this study.

2.6 Preliminary Phytochemical Screening of extract
Preliminary phytochemical analysis was carried out to check and identify the active constituents of the methanolic extract of *Achyranthus Aspera* leaves such as alkaloids, carbohydrates, flavonoids, terpenes, steroids, saponins and tannins by using test methods of Draggendroff's, Mayer's test, Molisch's, Fehling's test, lead acetate, Liebermann-Burchard test, foam formation test, ferric chloride test and Alkaline Reagent Test (Ramon 2006) [12].

Table 2: Qualitative chemical analysis of *Achyranthes aspera* extract

Phytochemicals	Phytochemical Test	*Achyranthus aspera* Extract
Alkaloids	Hager's test	+
Saponins	Frothing test	+
Phytosterols	Liebermann-Burchardt's test	+
Phenolic compounds	Ferric chloride test	+
Tannins	Lead acetate test	_
Phlobatannin	Hydrochloric acid test	-
Flavonoids	Alkaline Reagent Test	-
Detection of Free sugar	Fehling's Reagent test	-
Detection of Glycosides	Kellar - Kiliani test	-

3. Acute toxicity study
Limit test was performed as per OECD guideline for testing of chemicals (OECD, 2001) [10] to evaluate the acute oral toxicity of AAE in female albino rats with the upper limit dose of 2000 mg/kg. The mortality, behavioral abnormality, signs and symptoms of toxicity, if any, were recorded for a period of 14 days of post administration.

4. Antibacterial activity
The antibacterial activity of different extracts was studied by Disc Diffusion Technique Bauer *et al*. (2014) [2]. The blank discs of 6.25 mm diameter were punched from filter paper. The blank discs were weighed. Methanolic extract of AAE were prepared at the concentrations of 100 mg/ml, 200 mg/ml and 500 mg/ml in distill water and were used for the preparation of extract impregnated discs of extract. Then, each disc was impregnated with a particular concentration of an extract and then the weight of five discs impregnated with the extracts was again measured to calculate the net content of each extract in each disc for different concentrations.
Each of four bacteria *viz*. *E. coli, Staph. aureus, S. gallinarum* and *P. aeruginosa* was grown in nutrient broth, incubated at 37 °C overnight and diluted (10^{-5}) using sterile nutrient broth. Broth culture of each bacterium was spread over the nutrient agar taken in petri-dishes. The extract impregnated discs and a reference antibiotic (Ciprofloxacin) disc inoculated nutrient agar in the petri-dishes and incubated at 37 °C for 24 hour. The petri-dishes were observed for the presence of zone of inhibition around the discs and the zones of inhibition were compared with the reference antibiotic (ciprofloxacin) to assess the antibacterial activity of each extract.

5. Preparation of antigen
Fresh Sheep blood was collected in sterile Alsever's solution in 1:1 proportion and Kept in, refrigerator. Sheep red blood cells (SRBCs) for immunization were prepared by spinning

sheep blood at 2000rpm for 10 minutes, residue obtained after centrifugation was washed thrice in Normal saline solution (NSS) and finally a 7% suspension of SRBC was prepared Patel *et al*. (2010) [11].

6. Immunological study

6.1 Determination of Humoral Immune Response

Humoral immune response was assessed by micro haemagglutination test according to the method of Thaxton *et al.* (1974) [19] with minor modification. Each pretreated birds including the control group was immunized with 1ml suspension and injected intravenously to six birds (two from each replicate) from each group on 32 day of age and the birds were bled on day 10th day after injection. The blood was allowed to clot at 37 ºC for few hours and refrigerated and serum was collected. Serum was heated in a water bath to inactivate the complement fraction of the serum and Antibody production in response to the immunization was examined visually by micro-haemagglutination test. The highest serum dilution showing complete haemagglutination was noted and expressed as HA titer in $\log_2$ value.

6.2 Determination of Cell mediated immunity

Cell mediated immune response was measured by 2, 4-Dinitro-fluorobenzene (DNFB) test as described by Tamang *et al*. (1988) [18]. Featherless area was marked on both sides of abdomen. The area was cleaned thoroughly with acetone and air dried. Right lateral side of abdomen was used for DNFB application whereas left side served as control. 2000 µg of DNFB in 0.1 ml of acetone and olive oil (4:1) was applied on the right marked area on the abdomen using a plastic ring to avoid spillage. The sensitized birds were challenged with 50 µg of DNFB in 0.1 ml of acetone and olive oil (4:1) on the same area on day 14th after initial sensitization. The response to DNFB was assessed by measuring the skin thickness using engineer's micrometer on 0, 24 and 48 hours post challenge with three readings each and the overall mean skin thickness was calculated.

6.3 Statistical analysis

All the recorded data were subjected to statistical analysis as per standard methods and techniques described by Snedecor and Cochran (1994) [15] followed by comparison of mean values were further compared by Duncan's Multiple Range Test.

7. Results and Discussion

Phytochemical screening provide an empirical basis for the use of plants in traditional medicinal practices the biological or therapeutic activities of medicinal are closely related to their chemical compound. Phytochemical analysis of methanolic extract of *Achyranthus aspera* revealed the presence of alkaloids, glycosides, phytosterols, saponin and phenols. Results presented in (Table 2). The findings of this study is in agreement with the result of Kar *et al.* (2007) [7] and Saba Hassan (2014) [5].

In-vitro screening of the antimicrobial activity of the plant extract from *Achyranthus aspera* was studied against micro-organisms. *Salmonella gallinarum* and *Escherichia coli* showed sensitivity to AAE; at the level of higher concentration 500mg/ ml maximum zone of inhibition was 20.60 mm and 20.10 however *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed resistance to AAE even at this higher 500 mg/ml concentrations. The lower concentrations 100 mg/ml and 200 mg/ml did not show any antibacterial activity against *Salmonella gallinarum*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed resistant to standard antibiotic ciprofloxacin at these concentrations presented (Table 3). The results of present study is agreement with the Hossain *et al.* (2013) [6] who reported that antibacterial activity of *Achyranthes aspera* leaf methanol extract at concentration of 2 mg/disc and 3 mg/disc against *Bacillus cereus*, *Pseudomonas sp*., *Salmonella typhae* and Cefixime (5µg/disc).

Table 3: Zones of inhibition of bacteria shown by plant AAE extract and ciprofloxacin

Name of Extract	Extract in Each Disc (mg)	Zones of Inhibition (mm) ± SE			
		Staph. Aureus	***S. gallinarum***	***P. aeruginosa***	***E. coli***
Achyranthes aspera extract (100mg/ml)	3.00	6.35 ± 0.15 (R)	6.55 ± 0.25 (R)	6.60 ± 0.11 (R)	7.00 ± 0.20 (R)
Achyranthes aspera extract (200mg/ml)	4.60	6.55 ± 0.25 (R)	7.05 ± 0.25 (R)	6.50 ± 0.15 (R)	6.85 ± 0.35 (R)
Achyranthes aspera extract (500mg/ml)	6.00	6.55 ± 0.35 (R)	20.60 ± 0.20 (S)	7.35 ± 0.15 (R)	20.10 ± 0.20 (S)
Ciprofloxacin	5mcg /Disc	9.50 ± 0.51 (Resistance)	22.50 ± 0.51 (Sensitive)	13.50 ± 0.51 (R)	21.50 ± 0.52 (S)

In cell mediated immunity study, broiler chicks were exposed to the challenge dose of DNFB revealed erythema, oedema, vesiculation and scab formation. The effects of the *Achyranthus aspera* powder on cell mediated immune response T1, T2 and T3 were 1.78 ± 0.72^{b} 1.91 ± 0.78^{ab} and 2.21 ± 0.08^{a} at 24 hours 2.06 ± 0.08^{b} 2.08 ± 0.17^{b} and 2.75 ± 1.12^{a} at 48 hours are presented in (Table 4 and Table 5) Group T3 showed only stimulation of CMI significantly (p<0.05) higher skin thickness in DNFB skin sensitization test both at 24 hours and 48 hours after sensitization The HA titers of groups T1, T2 and T3 were 5.50 ± 0.67, 5.30 ± 1.23and 4.60 ± 0.21 but did not show any significant (p<0.05) effect on humoral immune response in broiler birds. Standard AGP (BMD) did not have any influence on the humoral as well as cellular immune status of broiler birds in this study. This indicated that *achyranthus aspera* powder @ 20 g/kg of feed continuously daily for 42 days caused enhancement of cellular immunity. The results of present study is also agreement with Shewita and Toha, (2011) [14] who reported that there was no significant difference in antibody. However contrary to present study Sharma and Singh (2016) [13] who reported the increment antibody titer in mice with root and leaf extract of *Achyranthes aspera* at the level of 100 and 200 mg/kg body weight due to the stimulation macrophage and B-lymphocyte.

8. Conclusion

The antibacterial activity and immunomodulatory effect observed in this study of *Achyranthus aspera* might be due to the presence of many potent phytochemical compounds such as saponin, Tannin phenolics and alkaloids etc. It can be concluded that the *Achyrantus aspera* extract can be used for protection against gram positive and gram negative bacteria

and also they are able to stimulate the cell mediated immune response. This research indicates further studies on the plants to isolate and identify the responsible biological active compounds in this plant.

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