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Brine shrimp bioassay as a preliminary assessment tool for development of herbal growth promoter formula for poultry

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ABSTRACT

Growth promoters play an important role in the animal agriculture industry as they facilitate to improve productivity mainly through reduction of pathogenic bacterial load in the gut. Ban and restrictions on the usage of Antibiotic Growth Promoters (AGP) due to public health concerns has made it essential to search alternatives from the herbal sources. But at the same time it is tricky to choose the best active molecule or combination from the wide range of herbs and extracts available. In this context, there is a requirement to have a simple bench top bioassay tool to screen these active molecules. Brine shrimp is one such method proposed as a simple bioassay tool for natural product evaluation. The procedure determines LC_{50} values in µg/ ml of actives in the brine media. Activities of a broad range of known active compounds are noticed as toxicity to the shrimp. In the present study, brine shrimp bioassay model has been adopted to evaluate the bioactivity of lipid soluble and water soluble fractions of garlic and found that the former was potent with LC_{50} value of 23.9µg/ml. Brine shrimp bioassay was also conducted on the formulation made using the above mentioned bioactive lipid soluble fraction of garlic as a major ingredient and found to be bioactive with LC_{50} value of 179.1µg/ml. The same was further confirmed in an In vivo trial conducted in poultry in comparison with popular AGP.

Keywords: Brine shrimp; Garlic; Bioactivity

INTRODUCTION

Antibiotics are used frequently in modern animal agriculture practice for the treatment of individual animals for specific disease conditions or as additives to feed and water to promote weight gain and improve feed conversion efficiency [1]. Of these, tetracyclines are most widely applied to food-producing animals as drugs and feed additives because of their broad spectrum activity. Excessive use of antibiotics result in residues in animal agricultural products and in turn may increase risk of resistance in human pathogens [2]. Residues of tetracycline compounds in poultry products in the eastern province of Saudi Arabia [3] and residue contamination of the meat products due to usage of large amount of antibiotics including Oxytetracycline and Sulfadiazine [4] are few instances of impact of residual contamination in the animal products.

Recent implementation of total ban on antibiotics as growth promoters in the feed in Europe is one indication of menace involved in the usage of antibiotics [5]. This triggers the quest for alternate source of growth promoters, probably from herbal origin. The choice of herb or herbal extracts is enormous and selection would be difficult unless there is a tool to evaluate the bioactivity of these natural products. Brine shrimp bioassay is one such general bioassay tool employed to screen wide range of compounds for various applications such as analysis of pesticide residues, mycotoxins, stream pollutants, anaesthetics, dinoflagellate toxins, morphine – like compounds, active phytoconstituents from higher plants and plant extracts etc [6]. But for development of pharmaceutical preparations with naturally derived active ingredients this methodology is less explored. In this study, an attempt was made to develop a growth promoter formula from herbal source using Brine shrimp as a preliminary bioassay tool. Chosen water soluble and Lipid soluble fractions [7, 8] of garlic were taken for evaluation using Brine shrimp bioassay and the component which showed better LC_{50} was included as a major component in the growth promoter formula. The efficacy of the formulated growth promoter was also screened using Brine shrimp bioassay in order to understand its bioactivity. Further, an *In vivo* trial was conducted in broiler chicks using the selected combination in comparison with popular AGP to confirm its ability to reduce the pathogenic bacteria in the target species.

MATERIALS AND METHODS

Extracts:
Lipid soluble & water soluble fractions of garlic extracted, purified and processed in the laboratory.

Formulation
The formulation was prepared by incorporating Lipid soluble fraction of garlic – Not less than 0.5 % with an inert carrier.

Test organism:
24 hours nauplii of the Brine shrimp *Artemia salina* (Sanders Brine Shrimp Company, USA) were used [9].

Bioassay:
Hatching of shrimps
Around 1 mg of dried eggs (Sanders Brine Shrimp Company, USA) were hatched in a dual glass chamber setup inter connected with a flexible transparent tube, filled with Artificial Sea Water (ASW) [9]. One chamber of the setup was closed with aluminium foil and the other portion was exposed to light at 28 ± 2 ºC. The phototrophic nauplii were collected as they swam out through the tube towards the chamber which was exposed to light.

Test procedure:
The bioassay was performed in specially fabricated narrow test tubes of 20 ml capacity. 20 nauplii were pipetted into each tube along with a measured quantity of ASW. The known concentrations of test samples were added to each tube and volume was adjusted to 20 ml with ASW. Potassium dichromate was maintained as positive control [9]. Negative control was maintained with the solvent used for test sample preparation. The percentage mortality was determined by comparing the mean surviving larvae of the test and control tubes. LC_{50} values were determined with 95% confidence intervals using Environmental Protection Agency (EPA) probit analysis program (Version 1.5) [10].

RESULTS AND DISCUSSION

The water soluble and lipid soluble fractions of garlic were subjected to repeated testing to arrive at a concentration range where 50% mortality of shrimp was observed. Experiments were repeated in triplicates over the selected concentration range for confirmation of their bioactivity. The lipid soluble fraction was found to be more potent with LC_{50} value of 23.9µg/ml, whereas water soluble fraction was found to be comparatively ineffective with an insignificant LC_{50} value of 18000µg/ml. The % Mortality of brine shrimp larvae after 24 hours of exposure to selected concentration range of lipid and water soluble fractions of garlic in triplicates are tabulated in Table 1 & 2 respectively.

Table 1: Showing % mortality of brine shrimp for lipid soluble fraction of garlic

Concentration (µg/ml)	Total number of larvae number exposed (20 no's x 3 replicates)	% Mortality
16	60	20
24	60	60
32	60	70
40	60	75
48	60	80

Table 2: Showing % mortality of brine shrimp for water soluble fraction of garlic

Concentration (µg/ml)	Total number of larvae number exposed (20 no's x 3 replicates)	% Mortality
10000	60	10
12000	60	30
14000	60	35
16000	60	40
18000	60	50

A growth promoter formula was prepared with the selected bioactive lipid soluble fraction as major ingredient. The formulation was subjected to repeated testing to arrive at a concentration range where 50% mortality of shrimp was observed. Experiments were repeated in triplicates over the selected concentration range for confirmation of its bioactivity. The LC_{50} value of the formulation was found to be 179.1µg/ml. The % Mortality of brine shrimp larvae after 24 hours of exposure to selected concentration range of formulation, estimated LC/EC Values and Confidence Limits are shown in Table 3 & 4 respectively. The formulation was further tested *In vivo* in broiler chickens using the selected combination along with popular AGP containing Virginiamycin as active ingredient to confirm its ability to reduce the pathogenic bacteria in the target species. The trial was conducted for a period of 42 days in the broiler chickens (Cobb 400) by supplementing these formulations in the basal diets. Negative control group (T1) was given only basal diet. The second (T2) and third (T3) groups were fed with starter/grower/finisher diet fortified with popular AGP (Virginiamycin) and formulation respectively. The experiment was conducted in the Provimi Research Centre, Doddaballapur (PRCD, CPCSCEA Registration No. 961/c/06/CPCSEA) as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The pathogenic bacteria isolated from the respective groups at the end of the trial were *Salmonella species* and *Escherichia coli*. Significant reduction was noticed in the average pathogenic bacterial counts in the isolates of chicken duodenum in T3 group. The results showing average pathogenic bacterial counts from 6 chicken duodenal contents isolated at 42 days of age from respective treatment groups are tabulated in Table 5.

Table 3: Showing % mortality of brine shrimp for the formulation

Concentration (µg/ml)	Replicates	Total number of larvae number exposed (20 no's x 3 replicates)	% Mortality
140	3	60	30
160	3	60	36.7
180	3	60	50
200	3	60	57
220	3	60	73.4

Table 4: Estimated LC/EC Values and Confidence Limits for the formulation

Point	Exposure concentration µg/ml	95% Confidence limit	
		Lower	Upper
LC/EC 1.00	69.287	38.602	90.329
LC/EC 5.00	91.508	60.360	110.714
LC/EC 10.00	106.137	76.540	123.506
LC/EC 15.00	117.311	89.782	133.061
LC/EC 50.00	179.094	168.076	191.178
LC/EC 90.00	302.200	258.986	421.728
LC/EC 95.00	350.512	288.891	534.813
LC/EC 99.00	462.922	354.070	836.300

Table 5: Showing average pathogenic bacterial counts from the chicken intestinal contents collected at 42 days of age from respective treatment groups

Treatments	*Salmonella* species (cfu /g)	*Escherichia coli* (cfu /g)
Negative control (T1) Only Basal diet	73333	46666
Basal diet with Popular AGP containing Virginiamycin (T2)	10000	800
Basal diet with formulation (T3)	566	3733

CONCLUSION

Many a studies have proved that brine shrimp larva assay as a simple and rapid toxicity test for plant products [11-14]. Even though it is not linked well with any biological activity, it can be considered as a preliminary bioassay tool for screening of compounds of plant origin, one such attempt was made in the present study. Brine shrimp bioassay tool was adopted in order to develop an efficient formula for growth promotion in poultry and in the present study it turned out to be a useful and convenient way to determine the bioactivity of a formulation. The formulation was later subjected to more elaborate In-vivo trial in broiler chickens and found to be a potent formulation in reducing the pathogenic bacteria. This supported the data obtained from the brine shrimp bioassay. This study indicates that brine shrimp bioassay can be utilised as a basic tool for selection of active fractions and combinations of herbal origin for various biological activities. Nonetheless additional research is required by testing different extracts, individual or in combination for various biological activities using brine shrimp bioassay to support the statement. In addition to this it can be considered as a routine bioactivity determination tool, as chromatographic and other related techniques does not give assurance in terms of biological activity of an individual drug or formulation. More over in a feed additive manufacturing unit it is not feasible to carry out *in-vivo* pharmacological tests on animals for every batch due to cost, time and animal ethical concerns.

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