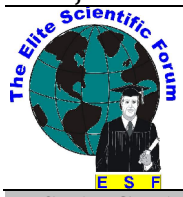
Pak. j. life soc. Sci. (2013), 11(3): 200-207 E-ISSN: 2221-7630;P-ISSN: 1727-4915



Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

RESEARCH ARTICLE

Saccharum officinarum Derived Mid Molecular Mass Glycoproteins as Native BRMs in Chickens

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ARTICLE INFO
Received: Aug 15, 2013
Accepted: Sep 23, 2013
Online: Oct 10, 2013

Keywords
Biological response modifiers
Chickens
Mid molecular mass glycoproteins
Saccharum officinarum

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ABSTRACT

Present study describes the biological response modifying (BRM) activities of *Saccharum officinarum* (sugar cane) derived mid molecular mass glycoproteins (MMMGs) in broiler chickens. MMMGs were isolated from sugar cane juice by size exclusion chromatography. The electrophoretic analysis of MMMGs by using Experion™ automated electrophoresis station showed the presence of thirteen different glycoprotein fractions with molecular weights ranging from 20.16 to 72.06 kDa in the isolated sample. BRM activities of MMMGs were evaluated in terms of cellular and humoral immune responses; and production performance. Findings of present study revealed that MMMGs significantly enhanced ($P<0.05$) the *in vivo* and *in vitro* lymphoblastogenic responses to Phytohaemagglutinin-P and Concanavalin-A, respectively in chickens. Significantly elevated B-cell mediated immune response in terms of antibody titres to sheep erythrocytes, Newcastle disease and Infectious bursal disease vaccines ($P<0.05$) in MMMGs administered chickens were also detected. Lymphoid organ-body weight ratios were statistically similar ($P>0.05$) in both MMMGs administered and control groups. Production performance was determined in terms of feed conversion ratios that was significantly improved ($P<0.05$) in chickens administered with MMMGs as compared to those of control group. In conclusion, MMMGs demonstrated immunostimulatory potential in broiler chickens and can be used as native BRMs in chickens to boost up their immune responses and production performance.

INTRODUCTION

During the last few decades, a variety of bioactive molecules has been isolated from different plants and fungi (Han et al., 2001). Studies on these biomolecules have demonstrated their potential of being the excellent biological response modifiers (BRMs) in both human and animal models (Ramberg et al., 2010; Paulson, 2001). The diverse biological activities of these biomolecules have attracted the attention of researchers to explore the possibility of their use in biomedical arena for their immunomodulatory and immune- therapeutic activities in different ailments. Thus, search and discovery of novel polysaccharides with different pharmacological properties may prove a great breakthrough in the field of biomedicine as it will be helpful in avoiding the drawbacks associated with irrational use of synthetic compounds (antibiotics) that results in the emergence of drug resistant strains, environmental pollution and residual effects in animal products being utilized by the human beings (Delespaux and Koning, 2007; Reig and Toldra, 2008). Findings of the previous studies have shown that potentiation of host defense mechanism(s) might be a possible means of inhibiting the growth of micro-organisms and tumors without harming the host (Ooi et al., 2000). Therefore, now a day, the goal of biomedical researchers is to discover and identify new safer drugs with no/minimal adverse effects.

In such circumstances, BRMs from plant origin with immunostimulatory acivities have been proven to be

suitable and promising alternatives to conventional chemotherapeutic agents (Patwardhan and Gautum, 2005). Now a day, natural products are becoming more imperative as efficient sources of pharmacotherapeutics, either as herbal remedies for treatment of chronic ailments or as raw materials from which active ingredients having particular BRM activities can be isolated. Due to the reason, more than 60% of newly approved anti-cancerous and anti-infective drugs are derived from natural sources (Schwartsmann et al., 2002; Harvey, 2008). Further, recent developments in the understanding of cellular and molecular basis of immune responses and complicated networking of immune system with other body systems has explored the importance of immunomodulation as a prophylactic or therapeutic strategy in maintaining a healthy state that has so far been desired.

To date, a number of plant derived components have been identified which are capable of boosting both the specific and non-specific natural immune responses. In this regard, sugar cane is an excellent source of bioactive molecules including falvones, policosanol, simple and complex sugars, sterols and phenolic compounds etc. (Takara et al., 2002; Mendoza et al., 2003). Biological and immunological activities of sugar cane derived constituents and extracts have been exhibited in different animal studies. These include anti-thrombotic (Molina et al., 2000), anti-inflammatory (Ledon et al., 2003, 2007), anti-oxidant (Nakasone et al., 1996; Takara et al., 2002), anti-stress (Brekhman et al., 1978) and immunomodulatory activities viz. adjuvant effects on the immune responses (El-Abasy et al., 2002, 2003a); protective effects against avian coccidiosis (El-Abasy et al., 2003b; Akhtar et al., 2008), endotoxic shock in mice (Motubu et al., 2006); radiation induced injury (Amer et al., 2004, 2005); and reconstituting effects on the B-cells in cyclophosphamide induced immunosuppression in chickens (El-Abasy et al., 2004). Keeping in view the diverse biological activities of sugar extracts, present study was conducted to evaluate the sugar cane derived mid molecular mass glycoproteins as native BRMs in industrial broiler chickens.

MATERIALS AND METHODS

Procurement and processing of Saccharum officinarum

Sugar cane (*Saccharum officinarum*) to be used in the present study was procured from Sugar cane Section, Ayub Agricultural Research Institute, Faisalabad, Pakistan and got authenticated by a botanist in the Department of Botany, University of Agriculture, Faisalabad (UAF), Pakistan. The plant specimens were kept in the Ethno-veterinary Research and Development Centre, Department of Parasitology, UAF as voucher No. 0171. Stalks from sugar cane plants were mechanically crushed in a cane crusher immediately after having been cut. Sugar cane juice (SCJ) thus obtained was centrifuged at 5000g for 15 minutes at 4°C. The pellet was discarded and supernatant thus obtained was subjected to filtration using membrane filters (0.45µm followed by 0.22µm) and stored at -20°C till further use for size exclusion chromatography.

Isolation and characterization of mid-molecular mass glycoproteins (MMMGs)

The filtered juice was subjected to size exclusion chromatography for the isolation of MMMGs by following the methodology described by Legaz et al. (1998) with minor modifications. Briefly, filtered juice was adjusted at a pH of 8.0 by adding saturated sodium carbonate (Na_2CO_3) solution followed by centrifugation at 4 °C (20000×g for 15 minutes). The sediment was discarded, and sodium azide was added to the supernatant to a final concentration of 0.02% (w/v). Clarified juice was chromatographed through Sephadex G-10 (Sigma®, USA) (15 × 2.5 cm), pre-equilibrated with saturated Na_2CO_3. Elutions were carried out with distilled H_2O and fractions (1.0 mL, each) were separated in Eppendorf tubes and analyzed for glycoproteins by taking optical density (OD) profile at 280 nm on ELISA reader (BioTek-MQX200, USA). Fractions at first two peaks obtained in profile were pooled together and considered as a mixture of glycoproteins. After separation of a sufficient quantity of required fractions, their mixture was chromatographed through Sephadex G-50 column (Sigma®, USA) (30 × 2.5 cm), pre-equilibrated as described above. Fractions (1mL) were subjected to ELISA reader in a 96 well flat bottom microtitration plate (Flow Labs., UK) and their absorbance was taken at 280 nm. The fractions in central peak(s) were considered for having MMMGs, lyophilized separately and stored in screw capped bottles till further use. The MMMGs isolated from sugar cane were characterized by using Experion™ automated electrophoresis station (BioRad®, USA) for their quantification and determination of molecular mass (es) according to the manufacturer's instructions.

Experimental design

A total of 100 broiler chicks (1-day-old; Hubbard) were purchased from local market and reared under standard managemental conditions at Experimental shed, Department of Parasitology, UAF. All the chicks were vaccinated according to local vaccination schedule (Awais, 2010). After acclimatization of birds for a period of 5 days, chicks was randomly divided into 4 equal groups; namely E_1, E_2, E_3 and E_4 and were administered orally with the MMMGs by using oral gavage for three consecutive days i.e. day 5^{th}, 6^{th} and 7^{th} of age as per schedule.

Group E_1: MMMGs at a dose rate of 50mg/kg of b.wt.

Group E_2: MMMGs at a dose rate of 100mg/kg of b.wt.

Group E_3: MMMGs at a dose rate of 150mg/kg of b.wt.
Group E_4: Phosphate buffered saline (4 mL)

Evaluation of biological response modifying activities

The BRM activities of HMMGs were evaluated in terms of cell mediated and humoral immune responses. Cell mediated immune (CMI) response was evaluated by *in vivo* and *in vitro* lymphoblastogenic responses to Phytohemagglutinin-P **(**PHA-P) and Concanavalin-A **(**Con-A), respectively; whereas immunoglobulin titres to sheep erythrocytes, Newcastle disease (ND) and Infectious bursal disease (IBD) vaccines were considered as indicators of humoral immune responses.

Cellular Immune responses

Classic toe-web assay was used to quantify the *in vivo* lymphoblastogenic response to PHA-P as described by Corrier (1990); whereas, peripheral blood lymphocytes blastogenesis assay was used to elucidate *in vitro* lymphoproliferative response in both treated and untreated chickens according to the method described by Qureshi et al. (2000).

Humoral Immune responses

Sheep erythrocytes were used as T-cell-dependent non-pathogenic antigens to demonstrate the antibody response. Anti-SE antibody titers were detected by using microplate haemagglutination assay by following the methodology of Yamamoto and Glick (1982) with minor modifications (Qureshi and Havenstein, 1994). Briefly, on day 14th post administration of MMMGs, chickens were injected sheep erythrocytes (5%) via IM route (1 ml/chicken) followed by a booster of same dose at day 14th post primary injection. Blood was collected at day 5th and 10th post primary and secondary injections to separate the sera. All the sera samples were titrated against sheep erythrocytes for total Igs, IgM (mercaptoethanol-sensitive) and IgG (mercaptoe-thanol-resistant) anti-sheep erythrocytes antibodies.

Antibody titers to ND vaccine were measured by hemagglutination inhibition (HI) assay according to the methodology described by Alexander (1988); whereas, antibody titres to IBD vaccine was measured by using ProFLOK® IBD ELISA Kit (Synbiotics Corporation, USA). All the buffers and reagents used in the procedure were diluted and used according the instructions of manufacturer.

Development of lymphoid organs

Chickens from all the groups were individually weighed and killed humanely on day 42nd of age. Lymphoid organs including bursa of fabricius, thymus, spleen and caecal tonsils were removed and weighed; and per cent organ to live body weight ratios were calculated by using the formula described by Giamborne and Closser (1990).

Feed conversion ratios (FCR)

Chickens from all the groups were weighed on weekly basis post administration of MMMGs. Feed given to each group was also recorded on weekly basis and the data thus obtained was used to calculate the weekly FCRs.

RESULTS

Automated electrophoretic analysis of MMMGs

The electrophoretic analysis of mid molecular mass glycoproteins isolated through molecular sieving technique revealed the presence of thirteen different glycoprotein fractions in the sample with molecular weights ranging from 20.16 kDa to 72.06 kDa.

Biological response modifying activities

In vivo lymphoproliferative response to Phytohaema-gglutinin-P (PHA-P)

Maximum swelling response (mm±SE; 1.24±0.034) was observed at 24 hours post PHA-P injection in chickens of group E_2 administered with MMMGs (100mg/kg of BW) followed by those of E_3 (1.224±0.028), E_1 (0.982± 0.032) and control (0.632±0.040) groups, respectively; whereas, the difference in MMMGs administered groups was statistically similar ($P>0.05$). A similar response was observed at 48 and 72 hours post PHA-P injection (Figure 1a).

In vitro lymphoproliferative response to Concanavalin-A (Con-A)

In vitro CMI response was detected on day 7th and 14th post administration of MMMGs by the assessment of lymphoblastogenic response of chicken peripheral blood leukocytes to Con-A. On day 7th, a statistically higher ($P<0.05$) response was detected in chickens administered with MMMGs (100mg/Kg of BW) followed by those of group E_3 (150mg/Kg of BW), E_1 (50mg/Kg of BW) and control groups, respectively; whereas, the difference between E_2 and E_3 was statistically similar ($P>0.05$). Similar results were observed on day 14th post administration of MMMGs (Figure 1b).

Antibody response to sheep erythrocytes

Results showed that on day 5th and 10th post primary injection (PPI) of sheep erythrocytes, chickens of group E_2 administered with MMMGs (100 mg/Kg of body weight) showed the highest titers followed by those of E_3, E_1 and control groups, respectively. Similar response pattern was observed on day 5th and 10th PSI of sheep erythrocytes. A similar trend like that of total anti-sheep erythrocytes antibodies titers was observed for IgM anti-SRBC antibodies on day 5th PPI; and 5th and 10th PSI; whereas, on day 10th PPI chickens of E_3 showed higher IgM geomean titre (27.86) as compared to those of group E_2 (27.24). At day 5th PPI, IgG anti- sheep erythrocytes antibody titers were similar in chickens of groups E_2 and E_3 (21.11) followed by E_1 (10.56) and control group (9.19); whereas, on day 10th PPI a similar response like total anti- sheep erythrocytes antibody titers was observed. On day 5th and 10th PSI, a similar pattern of IgG geomean titres was observed like that of total anti- sheep erythrocytes antibody titers (Table 1).

Table 1: Antibody response (geomean titres) to sheep erythrocytes

Total anti-sheep erythrocytes immunoglobulin titers				
Group	Day 5 PPI	Day 10 PPI	Day 5 PSI	Day 10 PSI
E_1	27.86	32.00	42.22	48.50
E_2	64.00	64.00	73.52	84.45
E_3	55.72	55.72	64.00	73.52
E_4	27.86	27.86	36.76	36.76
Anti-sheep erythrocytes Immunoglobulin-M titres				
E_1	17.30	13.62	23.84	11.74
E_2	42.89	27.24	36.76	64.00
E_3	34.61	27.86	36.14	55.72
E_4	18.67	11.86	15.65	8.90
Anti-sheep erythrocytes Immunoglobulin-G titres				
E_1	10.56	18.38	18.38	36.76
E_2	21.11	36.76	36.76	20.45
E_3	21.11	27.86	27.86	17.80
E_4	9.19	16.00	21.11	27.86

PPI=Post primary injection; PSI=Post secondary injection; E_1 = Mid molecular mass glycoproteins @ 50mg/kg of BW; E_2= Mid molecular mass glycoproteins @ 100mg/kg of BW; E_3 = Mid molecular mass glycoproteins @ 150mg/kg of BW; E_4= Control

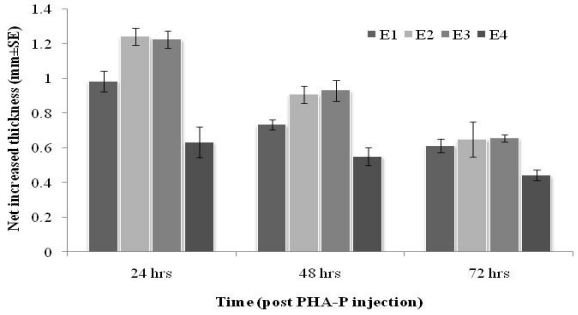
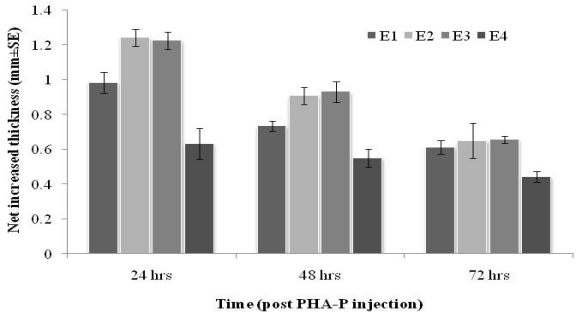


Fig. 1(a-b): Cellular immune responses to T-cell mitogens
a. Lymphoblastogenic response to Phytohaemagglutinin-P



b. Lymphoblastogenic response to Concanavalin-A
E_1 = Mid molecular mass glycoproteins @ 50mg/kg of BW; E_2= Mid molecular mass glycoproteins @ 100mg/kg of BW; E_3 = Mid molecular mass glycoproteins @ 150mg/kg of BW; E_4= Control

Antibody titers against ND and IBD vaccines

Humoral immune response was also detected against ND and IBD vaccines in terms of antibody titers. Results showed higher geomean titers against ND vaccine in MMMGs administered chickens, when compared with those of control group. Maximum geomean titer (256) was recorded in chickens of group E_2 (MMMGs@100mg/kg of BW) followed by E_3 (222.86), E_1 (194.01) and control groups (168.90). For antibody titers against IBD vaccine, chickens of group E_3 administered with MMMGs (150 mg/kg of BW) showed statistically higher ($P<0.05$) titers as compared to all other groups including control; whereas, difference of anti-IBD titers was statistically similar ($P>0.05$) between E_1 and control groups. (Figure 2a,b).

Lymphoid organs to body weight ratios

A statistically non-significant difference ($P>0.05$) was observed for the organ-body weight ratios in both MMMGs administered and control chickens (data not shown).

Effect on body weight gain and feed conversion ratios

The oral administration of MMMGs resulted in statistically higher ($P<0.05$) weight gains in treated chickens as compared to those of control group; whereas, the difference between MMMGs administered groups was statistically similar. On the other hand, chickens of group E_3 administered with MMMGs (150 mg/kg of BW) showed statistically better ($P<0.05$) FCR as compared to all other groups including control group (Figure 3).

DISCUSSION

Earlier, sugar cane extracts (SCE) have been reported for various biological activities including immunostimulation (Awais and Akhtar, 2012; Akhtar et al., 2008), anti-inflammatory (Ledon et al., 2007), vaccine adjuvant (El-Abasy et al., 2003a), anti-oxidant (Takara et al., 2002), anti-thrombosis (Molina et al., 2000), modulation of acetylcholine release (Barocci et al., 1999) and anti-stress (Brehkman et al., 1978) activities. Such findings demand for further investigations on sugar cane to identify the component(s) responsible for such activities. Keeping in view, present study was focused on the evaluation of BRM activities of MMMGs isolated from sugar cane juice so that their BRM activities may be exploited to minimize the use of chemotherapeutic drugs in poultry birds.

In current study, MMMGs induced up-regulation of cellular and humoral immune responses that highlighted the potential benefits of MMMGs in poultry industry. Higher i*n vivo* lymphoproliferative responses in chickens administered with MMMGs might be due to their stimulatory effects on the phagocytic activity of macrophages that led to an increase in the toe web thickness in response to T-cell mitogens (Akhtar et al., 2008). This response might also be correlated with enhanced delayed type hypersensitivity (DTH) response

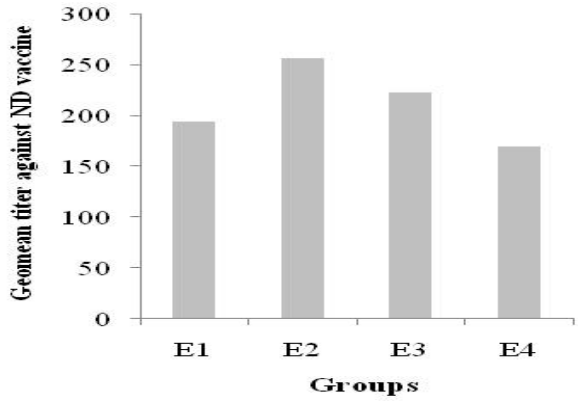
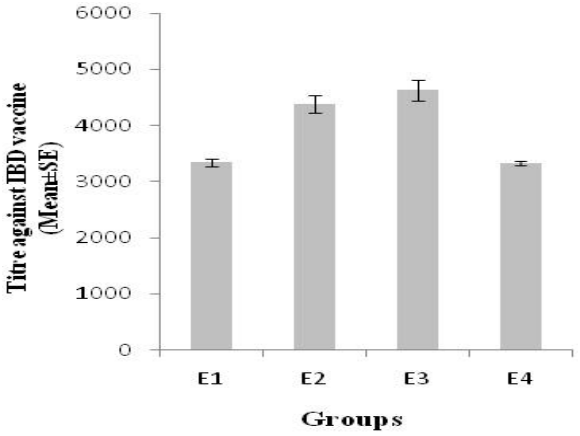


Fig. 2 (a-b): Antibody titers against ND and IBD vaccines

a. GMT against ND vaccine



b. Titres against IBD vaccine

E_1 = Mid molecular mass glycoproteins @ 50mg/kg of BW;
E_2= Mid molecular mass glycoproteins @ 100mg/kg of BW;
E_3 = Mid molecular mass glycoproteins @ 150mg/kg of BW;
E_4= Control

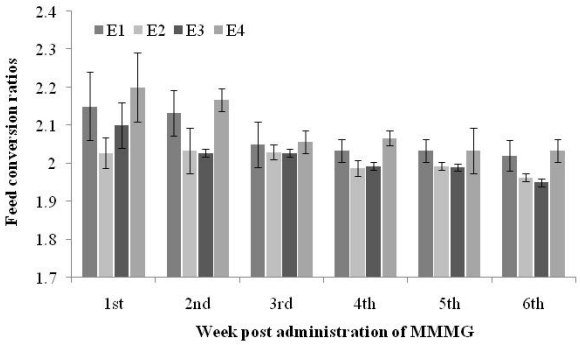


Fig. 3: Weekly feed conversion ratios (Mean±SE) post administration of MMMGs

E_1 = Mid molecular mass glycoproteins @ 50mg/kg of BW;
E_2= Mid molecular mass glycoproteins @ 100mg/kg of BW;
E_3 = Mid molecular mass glycoproteins @ 150mg/kg of BW;
E_4= Control

and magnitude of immune responses relies on the function and population dynamics of lymphocytes. Moreover, it may also be speculated that increased population of lymphocytes with high functional capabilities in the lymphoid tissues might be responsible for such immunostimulatory responses (El-Abasy et al., 2002). On the other hand, higher lymphoblastogenic response to Con-A might be correlated with mitogen receptors on T- lymphocytes present in the peripheral blood leukocytes that come in direct contact with the T-cell mitogen (Con-A) and the lymphocytes undergo cell division (Qureshi et al., 2000). Further, results of *in vitro* study were consistent with the findings obtained from *in vivo* experiment.

Significantly higher anti-sheep erythrocytes immunoglobulin titers in MMMGs administered groups indicated the higher humoral response suggesting that MMMGs had immunological properties to boost up the immunoglobulin production. This enhanced humoral response might be due to sugar cane factor (Pryce et al., 1990). Sugar cane derived polysaccharides had also been reported for stimulatory effects to activate the classical complement pathway in human serum by interacting with immunoglobulins (Li et al., 1983). Previous studies also revealed that oral administration of SCEs resulted in higher immunoglobulin responses to sheep erythrocytes in chickens initially infected with oocysts of *Eimeria tenella* (Awais et al., 2011; El-Abasy et al., 2003b); in radiation induced immunosuppressed chickens (Amer et al., 2004); increased serum antibody responses and number of antibody-producing cells in splenocytes, peripheral blood and intestinal leukocytes of chickens administered with SCE and polyphenol rich fraction of SCE (Hikosaka et al., 2007); and stimulatory effects of sugar cane juice on antibody production (Akhtar et al., 2008). Antibody responses to ND and IBD vaccines revealed that all the groups administered with MMMGs revealed higher geomean titres against ND and IBD vaccines as compared to control group. Similar to our findings, enhanced immune performance in terms of increased antibody titers to different vaccines by the use of medicinal plants and their components had also been reported in various studies. For instance, Sadekar et al. (1998) observed significant effect on the immune performance against IBD by the use of *Azadirachta indica*. Similar findings had also been reported by Akhtar et al. (2012) and Sarang (2005); whereas, Waihenya et al. (2002) reported a contradictory finding after the administration of crude extract of *Aloe secundiflora* in chickens in an immunological study on *Aloe*.

Feeding efficiency in terms of feed conversion ratios (FCRs) was also calculated and results showed that all the groups administered with MMMGs showed statistically improved FCRs as compared to control.

Improved FCR values in chickens administered with MMMGs indicated better feed utilization in experimental groups. The improved feeding efficiency might be due to the morphological and functional improvement of intestinal villi in response to SC derived glycoproteins (Yamauchi et al., 2006). Further, Awais & Akhtar (2012) and El-Abasy et al., (2004; 2002) also reported the similar findings that administration of SCEs into chickens resulted in higher body weight gains and better feed conversion ratios than those of control groups.

In conclusion, results of present study suggested that MMMGs derived from sugar cane had immune enhancing properties on both cellular and humoral arms of immunity. Additionally, these MMMGs had also positive impact on feeding efficiency in terms of improved FCRs. Further, supplementation of MMMGs as a native BRM in feed may be used to prevent and/or minimize the infections occurring in poultry industry.

Acknowledgement

The funds for this project were sponsored by Pakistan Science Foundation, Islamabad, Pakistan (Project No. P-AU/Bio (412)).

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