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RESEARCH ARTICLE

Saccharum officinarum Derived High Molecular Mass Glycoproteins as Native Biological Response Modifiers in Chickens

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A B S T R A C T

Present study reports the biological response modifying (BRM) activities of sugar cane (*Saccharum officinarum* L.) derived high molecular mass glycoproteins (HMMGs) in chickens. HMMGs were recovered from sugar cane juice by size exclusion chromatography. The electrophoretic analysis of HMMGs sample revealed the presence of eight different glycoprotein fractions with molecular weights ranging from 82.81 to 255.56 kDa. These HMMGs were evaluated for their BRM activities in terms of cellular and humoral immune responses and production performance. Results showed that HMMGs significantly enhanced ($P<0.05$) the *in vivo* and *in vitro* lymphoproliferative responses to phytohaemagglutinin-P and Concanavalin-A, respectively in chickens. Significantly elevated B- cell mediated immune response in terms of antibody titres to sheep red blood cells, Newcastle disease and Infectious Bursal disease vaccines ($P<0.05$) in HMMGs administered chickens were also detected. Production performance was determined in terms of feed conversion ratios that were significantly improved ($P<0.05$) in chickens administered with HMMGs as compared to those of control group. On the whole, results suggested that HMMGs have immunostimulatory potential and can be used as native BRM agents in chickens to boost up their immunity and production performance.

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INTRODUCTION

Immune system constitutes a remarkably sophisticated defense system in vertebrates that virtually participates in all processes of life from birth to death and protects them from various infectious and non-infectious diseases (Silin *et al*., 2009). In healthy individuals, it is capable to neutralize the invading pathogens successfully. However, infection develops only when the immune system is compromised or the load of pathogenic organisms is extremely high. Resultantly, the overwhelmed immune system leads to local or generalized colonization of infectious agents that interrupt the balanced physiology of the host (Baron, 1996).

There are various exogenous and endogenous factors which impair the activity of immune system leading to immunosuppression and mostly their elimination becomes impossible (Qureshi *et al*., 1998). These may include heat stress, mycotoxin exposure and certain immune-suppressive diseases (Awais *et al*., 2013). Under the circumstances, it is imperative to devise new ways and means to protect the immune balance of the animals in general and birds in particular to achieve maximum production potential. In this regard, a number of prophylactic approaches are being used to protect the immune competency including irrational use of chemicals/ antibiotics that resulted in the emergence of drug resistant strains, environmental pollution and residual effects in animal products being utilized by the human beings (Sahoo *et al*., 2010). In such circumstances, biological response modifiers (BRMs) from plant origin with immunostimulatory activities have been proven to be suitable and promising alternatives to conventional chemotherapeutic agents (Yoon *et al*., 2013). Now a days, 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 (Harvey, 2008).In this regard, a number of bioactive molecules including polysaccharides, glycoproteins, peptides, lectins and flavonoids extracted from different botanical sources have been identified for their immunomodulatory activities in different animal models (Cui *et al*., 2011; Thakur *et al*., 2011; Svajger *et al*., 2011).

Earlier, biological and immunological activities of crude sugar cane extracts have been exhibited in different animal studies. These include anti-thrombotic, anti-inflammatory, anti-oxidant and immunomodulatory activities viz. adjuvant effects on the immune responses (El-Abasy *et al*., 2002); protective efficacy against avian coccidiosis (Awais *et al*., 2011), endotoxic shock in mice (Motobu *et al*., 2006); radiation induced injury (Amer *et al*., 2005); and reconstituting effects on B-cells in immunosuppressed chickens (El-Abasy *et al*., 2004). In our previous studies, aqueous and ethanolic extracts from sugar cane juice and bagasse, respectively were evaluated to demonstrate their immunopotentiating and immunotherapeutic activities against avian coccidiosis (Awais *et al*., 2011; Awais and Akhtar, 2012; Awais *et al*., 2013). The encouraging results of this preliminary study compelled us to investigate the bioactive molecules present in sugar cane, responsible for such biological activities. Keeping in view, the current study was designed to evaluate the BRM activities of sugar cane derived glycoproteins in chickens.

MATERIALS AND METHODS

Procurement and processing of sugarcane: Sugarcane plants used in this study were procured from local market. After botanical authentication, plant specimen was kept in the Ethno-Veterinary Research and Development Centre, University of Agriculture Faisalabad-Pakistan (voucher No. 0171). Sugarcane stalks were crushed immediately after harvesting and juice was centrifuged (5000 g for 15 minutes at 4°C). Supernatant was filtered (0.22 µm membrane filters) and stored at -20°C till further use.

Isolation and characterization of glycoproteins: High molecular mass glycoproteins (HMMGs) were isolated from sugar cane juice by size exclusion chromatography (Legaz *et al*., 1998). Briefly, filtered sugar cane juice was adjusted to a pH 8.0 by adding saturated solution of Na_2CO_3 followed by centrifugation at 20000×g for 15 minutes at 4°C. Clarified juice was chromatographed through a Sephadex G-10 column (Sigma®, USA) (15×2.5 cm), pre-equilibrated with saturated Na_2CO_3. Elutions were carried out with DH_2O and analyzed for glycoproteins by taking optical density profile at 280nm 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 (1 mL) were subjected to ELISA reader in a 96 well microtitration plates (Flow Labs., UK) and their absorbance was taken at 280nm. The fractions in first peak were considered for having HMMGs, lyophilized separately and stored in vacuum dessicators till further use.

The HMMGs were characterized by using Experion™ automated electrophoresis station (BioRad®, USA) for their quantification and determination of molecular weight according to the manufacturer's instructions.

Experimental design: One-day-old broiler chicks (n=100; Hubbard) were purchased from local hatchery and reared under standard management conditions at Experimental shed, Department of Parasitology, UAF. All the birds were vaccinated according to local vaccination schedule. After 5 days of acclimatization, chicks were randomly divided into 4 equal groups viz. D_1 to D_4. Groups D_1, D_2 and D_3 were administered HMMGs @ 50, 100 and 150 mg/kg BW on 5^{th}-7^{th} days of age, respectively while Group D_4 was kept on phosphate buffered saline.

Evaluation of BRM activities: The BRM activities of HMMGs were investigated in terms of cellular and humoral immune responses. Cell mediated immune (CMI) responses were evaluated by *in vivo* and *in vitro* lymphoproliferative responses to Phytohemagglutinin-P **(**PHA-P) and Concanavalin-A **(**Con-A), respectively (Corrier, 1990; Qureshi *et al*., 2000). Humoral immunity was assessed by antibody responses to sheep red blood cells (SRBC), New Castle disease (ND) vaccine and Infectious bursal disease (IBD) vaccine. Antibody response to SRBCs was detected by using the methodology described by Qureshi and Havenstein (1994); whereas, antibody titers against ND vaccine were detected by indirect hemagglutination assay (Alexander, 1988). The titers against IBD vaccine were detected by using ProFLOK® IBD ELISA Kit (Synbiotics Corporation, USA).

Relative weights of lymphoid organs and Feed conversion ratios (FCRs): Chickens from all the groups were individually weighed and killed humanely at day 35^{th} post administration of HMMGs. Lymphoid organs were incised and weighed; and per cent organ-body weight ratios were calculated. Body weights and Feed consumption by each group were recorded on weekly basis to calculate weekly FCRs.

Statistical analysis: Data obtained were analyzed using ANOVA and LSD for the determination of statistical significance between experimental and control groups ($P<0.05$). Statistical differences for humoral responses against SRBCs and ND vaccine were determined by comparing geometric mean titers.

RESULTS

Automated electrophoretic analysis of HMMGs: The electrophoretic analysis of HMMGs isolated through molecular sieving technique revealed the presence of eight different glycoproteins in the sample with molecular

Table 1: Profile of high molecular mass glycoproteins isolated from sugar cane juice

Peak No.	Migration Time (sec)	Molecular Weight (kDa)	Corrected Area	Area ratio (To upper marker)	Concentration (ng/µL)
1.	16.85	1.20	1,064.20	Lower marker	
2.	20.16	5.69	1.58	System peak	
3.	21.08	6.93	258.30	System peak	
4.	36.84	82.81	125.79	41.1166	3083.74
5.	38.47	97.12	316.08	103.3139	7748.54
6.	39.95	111.40	30.30	9.9023	742.67
7.	41.63	128.06	2.70	0.8811	66.08
8.	44.95	163.66	3.65	1.1927	89.45
9.	46.07	177.60	6.67	2.1795	163.46
10.	50.41	231.48	15.93	5.2063	390.47
11.	52.34	255.56	2.30	0.7507	56.30
12.	52.70	260.00	3.06	Upper marker	

Table 2: Antibody response (geomean titers) to sheep Red Blood Cells

--------Total anti-SRBCs antibody titer--------				
Group	Day 5 PPI	Day 10 PPI	Day 5 PSI	Day 10 PSI
D_1	27.86	36.76	48.50	48.50
D_2	48.50	55.72	73.52	64.00
D_3	55.72	64.00	97.00	84.45
D_4	24.25	27.86	36.76	32
----------Immunoglobulin-M--------				
D_1	20.89	15.64	27.39	11.75
D_2	36.77	23.72	36.76	15.50
D_3	43.59	27.24	54.78	20.45
D_4	18.19	11.86	20.76	7.75
-------Immunoglobulin-G-----				
D_1	6.96	21.11	21.11	36.75
D_2	12.12	32.00	36.76	48.50
D_3	12.12	36.76	42.22	64.00
D_4	6.06	16.00	16.00	24.25

PPI=Post-primary injection to sheep red blood cells; PSI=Post-secondary injection to sheep red blood cells; D_1, D_2 and D_3= High molecular mass glycoproteins @ 50, 100 and 150mg/kg BW, respectively. D_4=Control

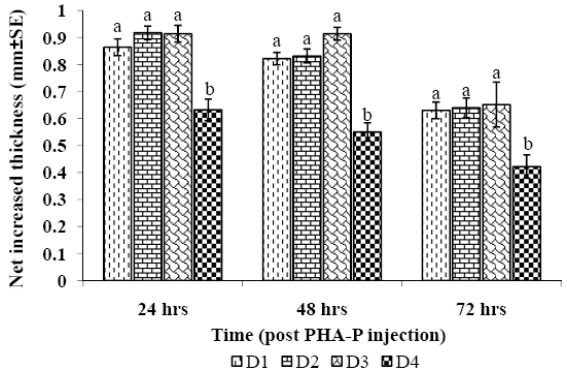
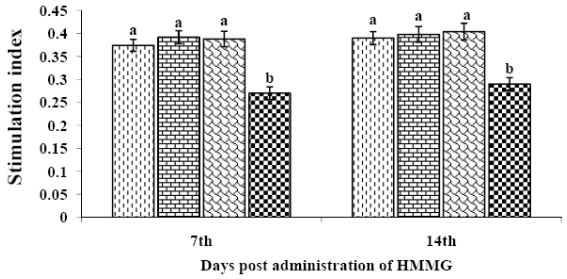


Fig. 1: Cellular immune responses to T-cell mitogens. ***a.*** *In vivo* lymphoproliferative response to phytohaemagglutinin-P



b. *In vitro* lymphoproliferative response to Concanavalin-A. Bars sharing similar letters on a particular time period are statistically non-significant (P>0.05); D_1, D_2 and D_3= High molecular mass glycoproteins @ 50, 100 and 150mg/kg BW, respectively. D_4=Control

weights ranging from 82.81 to 255.56 kDa. The molecular weights of constituent glycoproteins and their concentrations (ng/µL) are described in Table 1.

Biological response modifying activities

Cellular immune responses: Maximum *in vivo* lymphoproliferative response (mm±SE) to PHA-P in terms of toe web swelling (0.918±0.025) was recorded in group D_2 (HMMGs 100mg/kg of BW) after 24 hours followed by group D_3 (0.914±0.031), D_1 (0.864±0.031) and control group (0.632±0.040), respectively; whereas, statistical difference was similar (P>0.05) among all the experimental groups administered with HMMGs. The difference in response was statistically higher (P<0.05) in HMMGs administered groups, when compared with control group. A similar response was recorded at 48 and 72 hours post PHA-P injection (Fig. 1a). On both day 7th and 14th post administration of HMMGs, statistically higher (P<0.05) *in vitro* lymphoproliferative response to Con-A was detected in HMMGs administered chickens irrespective of the dose rate, when compared with those of control group (Fig. 1b).

Humoral immune responses: At day 5th post-primary injection (PPI), highest geometric mean anti-SRBC antibody titer (55.72) was detected in chickens administered with HMMGs (D_3; 150 mg/kg of BW) followed by groups D_2 (48.50), D_1 (27.86) and control (24.25). Similar results were observed on day 10th PPI. Moreover, at day 5th and 10th post-secondary injection (PSI), total anti-SRBCs Igs titres again showed same pattern as that on day 5th PPI. Similar trend was detected for anti-SRBCs IgM titers on days 5th and 10th PPI and PSI of SRBCs. At day 5th PPI, the highest IgG anti-SRBC antibody titers were detected in chickens of group D_2 and D_3 (12.12, each) followed by D_1 (6.96) and control (3.03), respectively. At day 10th PPI, chickens of group D_3 showed the highest response followed by those of group D_2 (32.00), D_1 (21.11) and control (16.00) (Table 2).

On the other hand, higher geomean titers against ND vaccine were detected in HMMGs administered chickens as compared to control group. Maximum geomean titer (256.00) was recorded in chickens of group D_3 (HMMG @ 150 mg/kg of body weight) followed by D_2 (256), D_1 (194.01) and control group D_4 (168.90). Further, chickens administered with HMMGs (150 mg/kg of BW) showed statistically higher (P<0.05) titers against IBD vaccine as compared to those of group D_1 and control group (Figure 2a-b).

Relative weight of lymphoid organs and FCRs: A statistically non-significant difference (P>0.05) was observed for the organ-body weight ratios in both HMMGs administered and control chickens (data not shown). Weekly FCR values were statistically improved/better (P<0.05) in chickens administered with HMMGs (150 mg/kg of BW) as compared to all other groups including control (Fig. 3).

DISCUSSION

In current study, *in vivo* cellular immune responses were evaluated by classical toe-web assay and results

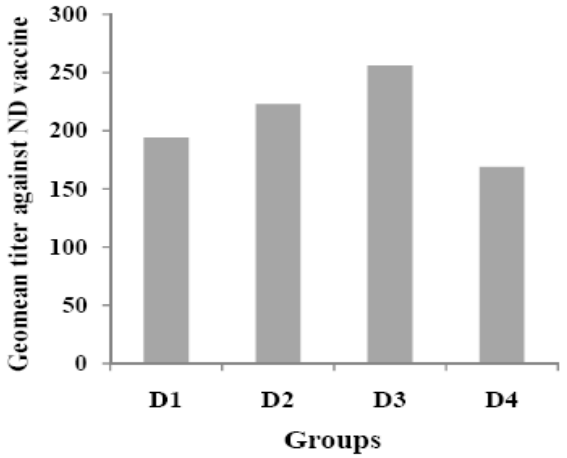
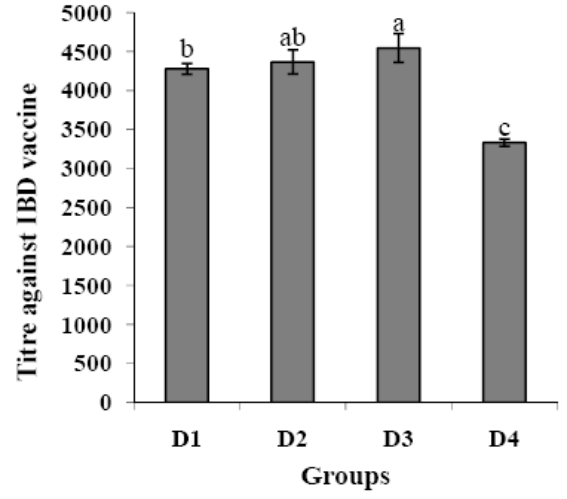


Fig. 2: (a-b): Antibody titers against ND and IBD vaccines. **a.** GMT against ND vaccine



b. Titres against IBD vaccine Bars sharing similar letters are statistically non-significant (P>0.05); D_1, D_2 and D_3= High molecular mass glycoproteins @ 50, 100 and 150mg/kg BW, respectively. D_4=Control

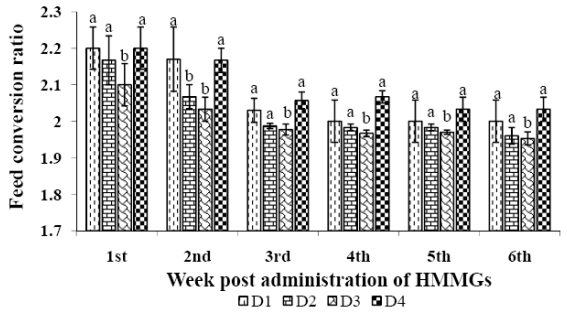


Fig. 3: Weekly feed conversion ratios (Mean±SE) post-administration of HMMGs. Bars sharing similar letters on a particular week are statistically non-significant (P>0.05); D_1, D_2 and D_3= High molecular mass glycoproteins @ 50, 100 and 150mg/kg BW, respectively. D_4=Control

showed statistically higher (P<0.05) response in birds administered with HMMGs as compared to control. The higher cellular immune responses in HMMGs administered birds might be due to their stimulatory effects on the phagocytic activity of macrophages that may lead to increased thickness of toe web in response to T-cell mitogens (Awais and Akhtar, 2012; Awais *et al*., 2013). It could be assumed that swelling of toe web in response to PHA-P mitogen in chickens might be due to enhanced delayed type hypersensitivity (DTH) and magnitude of immune response relies on the function and population of lymphocytes. Therefore, increased population of lymphocytes with high functional capabilities in the lymphoid tissues might be responsible for the stimulation of immune retorts (El-Abasy *et al*., 2002). On the other hand, higher lymphoproliferative response to Con-A might be due to the fact that the mitogen receptors on T- lymphocytes present in the peripheral blood leukocytes (PBL) come in direct contact with the T-cell mitogen (Con-A) and the lymphocytes go through cell division (Qureshi *et al*., 2000). Con-A stimulated the PBL which produced interleukine-1 by monocytes in PBL fraction, which stimulated the proliferation of lymphocytes (Abbas *et al*., 1991). Previously, similar immunostimulatory response of sugar cane extract on neutrophils to *Salmonella typhimurium* infection in mice has also been reported by Chen *et al*. (2012).

Results of humoral immune responses demonstrated that HMMGs administration resulted in higher total Igs, IgG and IgM titers against SRBC post primary and secondary injection of SRBC. Higher titers in experimental groups as compared to control indicated the higher humoral response that might be due to sugar cane factor (Pryce *et al*., 1990). Immunostimulatory effects of sugar cane derived polysaccharides on classical complement pathway in human serum by their interaction with antibodies had also been reported previously (Awais *et al*., 2011). Results of present study are consistent to the previous findings of similar studies that oral administration of sugar cane extracts resulted in higher antibody titers to SRBCs in chickens artificially inoculated with *Eimeria spp*. (El-Abasy *et al*., 2003); elevated antibody response to SRBCs in immuno-compromised chickens (Amer *et al*., 2005); stimulatory activity of sugar cane juice on immunoglobulin production (Akhtar *et al*., 2008) and stimulatory effect of aqueous and ethanolic extracts of sugar cane on immunoglobulin production (Awais and Akhtar, 2012).

Antibody responses to ND and IBD vaccines revealed that all the groups administered with HMMGs showed higher titres against ND and IBD vaccines as compared to control groups. 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 previous studies (Akhtar *et al*., 2012); 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*.

Weekly FCRs were almost similar in all HMMGs administered groups but observed better than control chickens; indicating improved feed utilization in HMMGs administered groups and a decrease in the amount of food needed for unit gain in body weight. Awais and Akhtar (2012) and El-Abasy *et al*. (2004) also revealed similar findings that oral administration of sugar cane extract resulted in higher body weight gain and feeding efficiency as compared to chickens of control group.

Conclusion: Results suggested that sugar cane derived HMMGs had biological properties of stimulating the both cellular and humoral arms of immunity as well as growth rates in industrial broiler chickens.

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