



THE ADJUVANT EFFECT OF *ZINGIBER OFFICINALE* EXTRACT AND SORBITAN MONOSTEARATE NIOSOMES ON LA SOTA® VACCINE IN BROILERS

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ABSTRACT

Newcastle disease is one of the most devastating viral diseases affecting poultry and economy. This research seeks to investigate the adjuvant effect of *Zingiber officinale* (ZO) and sorbitan monostearate (Span 60) niosomes on La Sota® vaccine in broilers. The vesicles of the niosomal La Sota® vaccine was characterized for particle size and zeta potential. Five groups of twenty birds each were evaluated for immune responses to Newcastle disease vaccine. One group was given *Zingiber officinale* as well as La Sota® vaccine encapsulated in sorbitan monostearate niosomes. The birds vaccinated with the ZO/Span 60 niosomal ND vaccine had the highest mean antibody titer of (\log_2) 8.20 ± 0.13 which was significantly higher than the live La Sota® group with (\log_2) 6.40 ± 0.56 . The unvaccinated groups did not produce any immune responses to Newcastle disease antigen. Nine out of ten birds in the unvaccinated group died while none of the birds died from the vaccinated groups. The stability of the ZO/Span 60 niosomal ND vaccine was also assessed through haematology and biochemical studies. The results showed stability at extreme conditions. Administering *Zingiber officinale* with La Sota® vaccine encapsulated in sorbitan monostearate niosomes could be a promising immunoenhancer for La Sota® vaccine.

KEYWORDS: La Sota® vaccine, Newcastle disease, Niosome, Sorbitan monostearate, *Zingiber officinale*

INTRODUCTION

The immune system of chicken has been studied extensively because of the need to increase food security and animal health. There is on-going interest in understanding the mechanisms of immunostimulation and developing strategies to enhance or hyper-stimulate responsiveness in poultry especially in countries where Newcastle disease is endemic due to low biosecurity [1]. The ultimate goal for researchers is to achieve a state of high resistance of the birds to either natural or experimental disease challenge. Modern research

Practices include vaccination designs which hyperimmunize hens to effectively transfer passive immunity to the progeny and initiate natural immunity from day of hatch. Furthermore, for the vaccines to be effective, it is important that they are delivered properly and effectively for maximum immunological benefit [2, 3]. Immunological adjuvants may make a valuable contribution in decreasing the minimum effective dose of antigen, thereby reducing the toxicity and expense of the vaccine, and increasing its availability [4]. Adjuvants may act by a combination of various mechanisms including formation of depot, induction of cytokines

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and chemokines, recruitment of immune cells, enhancement of antigen uptake and presentation, and promoting antigen transport to draining lymph nodes [5]. One can also exploit the use of specific dietary supplements to boost the intrinsic potential of poultry to perform better immunologically. Possible candidates for such dietary supplements include several vitamins, *Zingiber officinale*, garlic, *Garcinia kola*, *Vernonia amygdala*, etc. Researchers in vaccinology are also turning to advances in the worlds of micro- and nanotechnology by deploying particulate drug carriers including microparticles, nanocarriers, lipid-based carriers and colloidal carriers which encapsulate the antigen and direct them to specifically targeted parts of the body. Niosomes are non-ionic surfactant vesicles which are bilayered structures. Niosomes are chemically stable and can entrap both lipophilic and hydrophilic drugs either in aqueous layer or in vesicular membrane and present low toxicity because of their non-ionic nature. Niosomes present low production cost, greater stability and resultant ease of storage. Other advantages include flexibility in their structural constitution, improvement of drug availability and controlled delivery at a particular site [6]. This colloidal vaccine-loaded particles consists of macromolecular materials in which vaccines are dissolved, encapsulated and/or to which the vaccines are adsorbed or attached [6]. Dicetylphosphate, an anionic lipid was also included in the preparation of niosome to impart a negative charge and create charge stabilization of the colloidal system. In this study, ZO-primed sorbitan monostearate-based niosome was used to entrap a standard ND vaccine for possible enhancement of immunogenicity. The results suggest that the ZO-primed sorbitan monostearate based niosome might be suitable as a potential immunoenhancer/adjuvant for live La Sota® vaccine.

MATERIALS AND METHODS

Preparation of the niosome suspension

La Sota® vaccine (National Veterinary Research Institute, Jos, Nigeria), containing 200 doses/vial was reconstituted with phosphate-buffered saline by dissolving a vial in 40 ml phosphate buffer solution. Then 38.6 mg cholesterol, 43 mg sorbitan monostearate and 5.46 mg dicetylphosphate (all from SigmaAldrich Chemie, St Louis, Missouri, USA) in a 10:10:1 molar ratio were weighed and dissolved in 3 ml chloroform/methanol system (2:1) in a round-bottomed flask. The solvent mixture was evaporated at room temperature and the flask

rotated until a smooth, thin, dry film was obtained on the wall of the flask. A 5 ml volume of the reconstituted vaccine was used to hydrate the dry film and swirled clockwise rotation until multilamellar vesicles were formed.

Vaccination schedule of the birds

Day-old chicks (*Gallus gallus domesticus*) from CHI farms, Nigeria were used. The chicks were raised from day-old until termination of the experiment. All animal handling and experiments were conducted following the guidelines stipulated by University of Nigeria Research Ethics Committee on animal handling and use. One hundred birds were divided into five groups of 20 birds; the unvaccinated group, the ZO-primed niosomal ND vaccine group, the niosomal ND vaccine group, La Sota® vaccine group and the ZO group. The birds in the different groups were housed in separate rooms and all cleaning and feeding was planned to minimise the risk of cross-contamination. The ZO-primed niosomal ND group and the ZO group were given 0.5 ml of 10-fold dilution of *Zingiber officinale* extract daily for 2 weeks prior to immunization. At 2 weeks of age all the experimental birds were assessed for presence of maternal antibodies to Newcastle disease virus vaccine. At 3 weeks of age, the ZO-primed niosomal ND vaccine group was given 0.2 ml/bird (a dose) of the niosomal ND vaccine orally. The niosomal ND vaccine group was given 0.2 ml/bird (a dose) of the niosomal ND vaccine orally. The La Sota® vaccine group was given 0.2 ml/bird (a dose) of the reconstituted La Sota® vaccine orally. The unvaccinated group served as the control. Two weeks after primary vaccination, all birds were bled from the jugular vein and serum samples assessed for antibody to ND virus by the hemagglutination inhibition (HI) technique [7].

Booster vaccination was done at 6 weeks of age following the procedure for primary vaccination. Two weeks after secondary vaccination, all birds were bled and serum samples assessed for antibody to ND virus. Table 1 of vaccination schedule is seen below.

Stability studies using Haematology and blood biochemistry profile of the birds

Another set of hundred birds raised from day-old were divided into five groups of 20 birds; the unvaccinated group, niosomal ND vaccine group at room temperature, niosomal ND vaccine at freeze-thaw conditions, La Sota® vaccine group at room temperature, La Sota® vaccine group at freeze-thaw

conditions. Blood was collected after primary vaccination in ethylenediamine tetraacetic acid (EDTA) bottles for the following analyses:

Determination of total white blood cell count

A 0.02-ml volume of blood was pipetted into a small test tube containing 0.38 ml avian white blood cell diluting fluid to make a 1:20 dilution of the blood sample. The diluted sample was loaded onto the Neubauer counting chamber and all cells on the four corner squares were counted using a light microscope at 10 objective. The number of cells counted for each blood sample was multiplied by 50 to obtain the total white blood cell count per microliter of blood [8].

Determination of differential white blood cell count

The blood samples were shaken gently and a drop of blood was placed on clean grease free slide. The drop of blood was carefully smeared on the slide using a coverslip to make a thin smear. The smear was air-dried and thereafter stained by the Leishman technique using Leishman stain. The stained slides were later examined with an immersion objective using a light microscope. Two hundred cells were counted by the longitudinal counting method and each cell type was identified and scored using the differential cell counter. Results for the heterophils, lymphocytes, monocytes, eosinophils and basophils were expressed as a percentage of the total count and converted to the absolute value per microliter of blood [9].

Determination of alanine aminotransferases

A 0.5-ml volume of alanine aminotransferase substrate was incubated for 5 min at 378K. A 0.1-ml volume of the test samples were added and incubated at 378K for 30 min. Then 0.5 ml color developer was added and left to stand at room temperature for another 20 min, after which five milliliter of 0.4 N NaOH was added and left to stand for 15 min at room temperature. The readings were taken at a wavelength of 520 nm using a colorimeter [10].

Determination of total protein

A 0.02-ml volume of the samples was added to one milliliter Biuret reagent, mixed and allowed to stand for 10 min at room temperature. The readings were taken at a wavelength of 540 nm. Albumin and globulin were calculated.

Determination of uric acid

A 0.02-ml volume of the test samples was mixed with 1 ml working reagent (made ready to use). This was mixed and allowed to stand for 10 min at 35. The readings were taken at a wavelength of 505 nm.

Challenge experiment

The challenge experiment was carried out at 9 weeks of age in 10 birds from each group. The challenge was performed by administering 0.2 ml of $10^{5.5}$ median embryo lethal dose/ml (Herts-33 strain) to each bird by the oral route. After challenge, the birds were monitored for three weeks for clinical signs of disease and mortality. Chickens without clinical signs of ND were considered protected; those that showed the typical neurological signs or died within this period were considered not protected.

Data analysis

Data were fed into the SPSS statistics program (version 16.0; SPSS Inc.) applying a one-way analysis of variance test with least squared difference multiple comparisons at $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of the niosomal particles

The particle sizes of the sorbitan monostearate niosomes varied from 60 d.nm to 1000 d.nm Small ($<10 \mu\text{m}$) vesicles due to their large surface to mass ratio, are capable of facilitating extracellular delivery of antigen to the phagocytic accessor cells leading to faster release and increased antigen processing (Figure 1). A combination of larger and smaller particles might produce a pulsatile pattern for antigen release thus mimicking an immunization process involving prime and booster shot [11]. In other words, the size of the niosomal vesicles depend on the cholesterol content, charge incorporation or hydrophilicity of surfactants [12]. Sorbitan monostearate is non-ionic and hydrophilic in nature. The niosomes were prepared on a 50:50 sorbitan monostearate:cholesterol ratio which gives stable vesicles. Inclusion of cholesterol in niosomes increases its hydrodynamic diameter and entrapment efficiency [13]. It also leads to rigidity of the bilayers and therefore sustained release of the entrapped vaccine [14-16]. The zeta potential of the sorbitan monostearate-based niosomes was -46.6 mV (Figure 2). The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. The general dividing line between stable and unstable dispersions is generally taken

at either +30 or -30 mV. Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered

stable. The zeta potential of the niosomal preparation was -46.6mV which can be considered very stable.

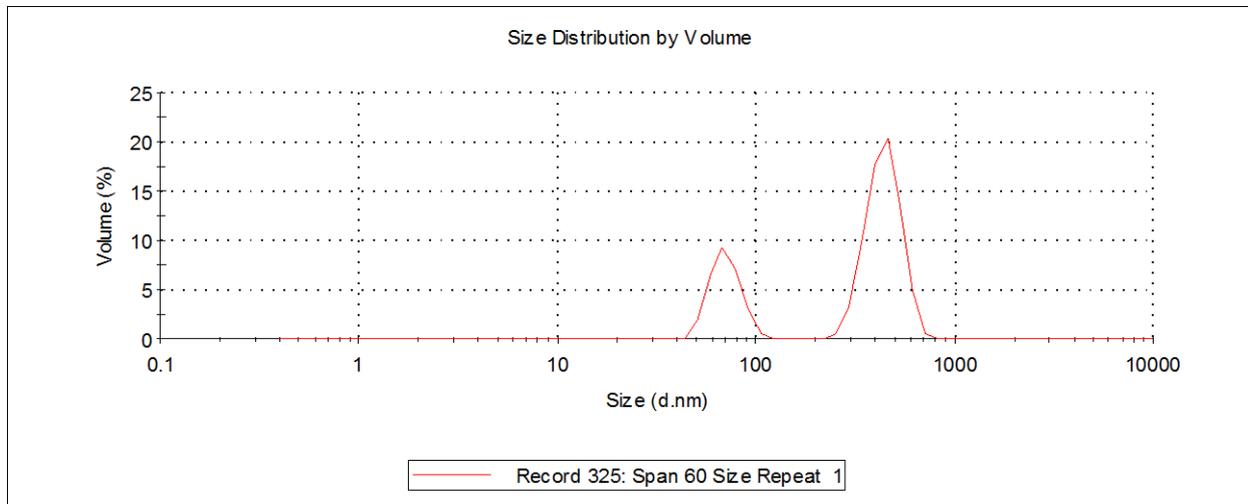


Figure 1: The particle size distribution of sorbitan monostearate niosomes

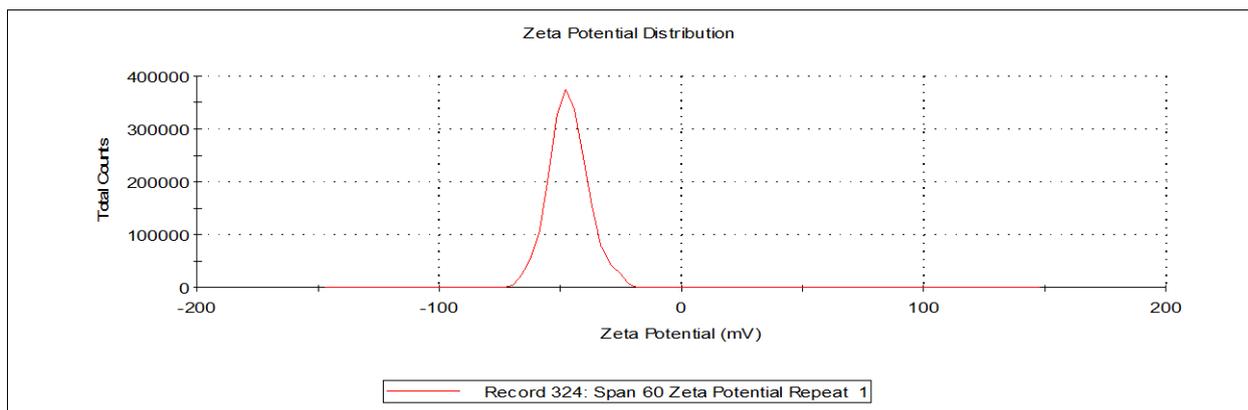


Figure 2: The zeta potential distribution of sorbitan monostearate niosomes

Table 1: Vaccination schedule

Batch	Priming with ZO	Primary vaccination	Secondary vaccination	Challenge with Herts 33 strain
Unvaccinated group	-	-	-	0.2 ml of $10^{5.5}$ median embryo lethal dose/ml
ZO group	0.5 ml	-	-	0.2 ml of $10^{5.5}$ median embryo lethal dose/ml
ZO-primed niosomal vaccine group	0.5 ml ND	0.2ml/bird	0.2ml/bird	0.2 ml of $10^{5.5}$ median embryo lethal dose/ml
Niosomal vaccine group	ND	0.2ml/bird	0.2 ml/bird	0.2 ml of $10^{5.5}$ median embryo lethal dose/ml
La Sota® vaccine	-	0.2 ml/bird	0.2 ml/bird	0.2 ml of $10^{5.5}$ median embryo lethal dose/ml

Table 2: Immune response of the birds to Newcastle disease virus vaccine formulations

Treatment group	Primary immunization *(log ₂)	Secondary immunization *(log ₂)
Negative control	0.00	0.00
ZO	0.40± 0.27	0.00
La Sota® vaccine	3.20 ±0.44	6.40± 0.56
ZO-primed niosomal ND vaccine	2.90± 0.48	8.20±0.13
Niosomal based ND niosomes	4.50± 0.54	6.40±0.34

*The antibody titres are expressed in log base 2

Table 3: Haematological profile of the birds after immunization using vaccines kept extreme conditions

Leucocytes x1000/ul	Total white blood cells	Heterophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Negative control (unvaccinated)	13.85 ±1.31	3.39±0.54	9.76±1.26	0.25±0.06	0.35±0.05	0.09±0.04
Niosomal ND vaccine (28 °C)	34.22± 3.00	9.81±2.15	24.28± 3.78	0.08±0.08	0.06±0.06	0.0
Positive control (LaSota®) 28 °C	26.71 ±2.98	5.15±0.57	21.11±2.70	0.21±0.10	0.10±0.06	0.00
Niosomal ND vaccine (7-freeze-thaw cycles)	26.71 ±1.79	6.88±1.58	19.06±0.75	0.22±0.06	0.43±0.16	0.12±0.07
Positive control (LaSota®) 7-freeze-thaw cycles)	26.69 ±2.69	10.89±1.65	16.89±1.67	0.17±0.07	0.50±0.17	0.05±0.05

Antibody responses

All birds screened prior to administration of vaccine were negative for HI antibody. All control (unvaccinated and ZO groups) birds had no antibody throughout the experiment. The immune responses of the birds are shown in Table 2. After primary immunization, the niosomal-based ND group had the highest mean antibody titre (log₂) of 4.50 ±0.54, the live La Sota® vaccine group had a titre of 3.20 ±0.44 while the ZO-primed niosomal ND vaccine group had an antibody titre of 2.90 ± 0.48. There was no significant difference among the vaccinated groups. After secondary immunization the chickens further seroconverted, and the ZO-primed niosomal based ND vaccine group had an antibody titre of (log₂) 8.20±0.13 which was significantly higher than the La Sota® group with

(log₂) 6.40 ± 0.56 or the niosomal based ND group with (log₂) 6.40±0.34.

The control groups (unvaccinated and ZO) did not produce any immune response to Newcastle disease antigen from the chicken. The increased titre value of the ZO-primed niosomal ND vaccine group shows an immunostimulating effect of ZO in the overall mean immune response titre. ZO and other immunomodulatory plants can alter the immune system of an organism by interfering with its functions, if it results in an enhancement of immune reactions; it affects granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances [17]. The target sites of the niosomes via the oral route are the M cells within the Peyer's patches along the small intestine. M cells are vital in establishing mucosal

immunity and allow a route for antigens to be delivered. This route delivers the antigen to underlying lymphoid tissues where a secretory immune response is initiated [18-20]. M cells remain good target sites for niosomes and other oral vaccine delivery systems. The antigen has to be physically associated with niosomes to produce adjuvant activity where by the antigen is incubated and endosomally encapsulated [21].

Challenge studies

Nine of ten challenged birds in the unchallenged group died while none died from the ZO-primed niosomal ND vaccine group or the niosomal ND vaccine group or the live La Sota® vaccine group. Post-challenge signs observed among the unvaccinated birds and the lesions observed at post-mortem of deceased chickens were identical with those described for ND [22].

Stability of vaccines

In Table 3, the results of the stability studies are seen using the haematological and blood chemistry profile of the birds as the parameter for assessment. The vaccines kept at different extreme conditions showed that the immune responses of

the vaccinated birds were significantly higher in the group with the sorbitan monostearate than La Sota® vaccine. In Table 4, is shown the blood chemistry profile of the birds after immunization. The highest number of circulating white blood cells x1000/ul was also produced by birds immunized with sorbitan monostearate niosomal ND vaccine at 28 °C. The result (Table 3) shows that the vaccine kept at 28 °C for five weeks were still stable. This result is particularly useful because of the extreme conditions which poultry farmers keep their vaccines especially in developing countries. It gives an indication of the potency of the vaccine after storage at untoward conditions. Formulating the Newcastle disease vaccine by encapsulating in sorbitan monostearate niosomes did not affect the stability even at extreme conditions. The presence of circulating lymphocytes in the blood shows a possibility of cell mediated immunity. Niosomal ND vaccine at seven freeze-thaw cycles still showed an appreciable level of circulating lymphocytes which was higher than the positive control (LaSota®vaccine) at the same condition. There was no appreciable toxicity detected from the biochemical tests as seen in Table 4 showing that niosomes are biocompatible and not toxic to the organs of the birds.

Table 4: Blood chemistry profile of the birds after immunization using vaccine kept extreme storage conditions

Treatment groups	ALT IU/L	Total proteins g/dl	Albumin g/dl	Globulin g/dl	Uric acid mg/dl
Negative control (unvaccinated)	6.18±1.51	2.88±0.07	1.22± 0.11	1.66± 0.14	2.50± 0.82
Niosomal ND vaccine (28 °C)	7.02±1.04	3.05± 0.17	1.38± 0.11	1.67± 0.12	4.25±1.55
Positive control (LaSota®) 28 °C	4.07± 0.34	3.01±0.08	1.19± 0.06	1.81±0.11	2.50±0.82
Niosomal ND vaccine (7-freeze-thaw cycles)	3.94±1.06	2.97± 0.04	1.08±0.09	1.89±0.08	3.38± 0.96
Positive control (LaSota) (7-freeze-thaw cycles)	3.68±1.31	2.87± 0.25	1.26± 0.06	1.61±0.06	2.00±0.37

CONCLUSION

From the results the La Sota® vaccine administered with *Zingiber officinale* and sorbitan monostearate niosomes holds good potentials for boosting both humoral and cell-mediated immune

responses. This hyperimmunity would reduce losses to Newcastle disease virus thereby promoting animal health and food security. The sorbitan monostearate is biocompatible and not toxic to the organs of the chicken as seen in the biochemical tests.

REFERENCES

1. Sharma JM (1997). The structure and function of the avian immune system. *Acta Veterinaria Hungaria*, 45(3): 229-238.
2. Koch G (1994). Immunological principles of vaccination of poultry: A lot to explore. In: *Advances in avian immunology research*. T.F. Davison, N. Bumstead, P. Kaiser, Eds. Carfax Publishing Co., Oxfordshire, UK. pp 197-206.
3. Baxendale W (1996). Current methods of delivery of poultry vaccines in Poultry immunology (Davison, T.F. Morris, T.R. Payne, L.N. Eds. Carfax Publishing Co., Oxfordshire, UK. pp 375-387.
4. Hobson D (1973). The potential role of immunological adjuvants in influenza vaccines. *Postgraduate Medical Journal*, 49(569):180-184.
5. Sunita A, Lorne B, Mutwiri G (2013). Mechanisms of action of adjuvants, frontiers in immunology. [Dx.DOI.org/10.3389/fimmu.00114](https://doi.org/10.3389/fimmu.00114).
6. Carafa M, Santucci E, Alhaique F, Coviello T, Murtas E, Riccieri FM, Luciana G, Torrisi MR (1998). Preparation and properties of new unilamellar nonionic/ ionic surfactant vesicles. *International Journal of Pharmaceutical Sciences*, 160(1): 51-59.
7. Office International des Epizooties (OIE) (World Organization of Animal Health). (2004). *Manual of Standards of Diagnostic Tests and Vaccines*.
8. Schalm OW, Jain NC, Carrol EJ (1975a). *Veterinary Haematology* (3rd Edn.). Lea and Febiger, Philadelphia, USA. pp 129-225.
9. Schalm OW, Jain NC, Carrol EJ (1975b). *Veterinary Haematology* (3rd Edn.). Lea and Febiger, Philadelphia, USA. pp 19-25.
10. Reitman S, Frankel, SA (1957). Colorimetric method for the determination of serum oxaloacetic and glutamic pyruvate transaminase. *American Journal of Clinical Pathology*, 28: 56-63.
11. Ruckmani K, Sankar V (2010). Formulation and optimization of zidovudine niosomes. *AAPS Pharmaceutical Sciences and Technology*, 11(3): 1119-1127.
12. Khandare JN, Madhavi G, Tamhankar BM (1994). Niosomes: novel drug delivery system. *The Eastern Pharmacist*, 37: 61-64.
13. Yoshioka T, Stermberg B, Florence AT (1994). Preparation and properties of vesicles (niosomes) of sobitan monoesters (Span 20, 40, 60, and 80) and a sorbitan triester (Span 85). *International Journal of Pharmaceutical Sciences*, 105: 1-6.
14. Yoshioka T, Stenberg B, Moody M, Florence AT (1992). Niosomes from Spansurfactants: Relations between structure and form. *Journal of Pharmacy and Pharmacology*, 44: 1044.
15. Yoshida H, Leh CM, Kok W, Junginger HE, Verhoef JC, Bouwistra JA (1992). Niosomes for oral delivery of peptide drugs. *Journal of Controlled Release*, 21: 145-153.
16. Zerrin Sezgin-Bayindir Z, Yuksel N (2012). Investigation of formulation variables and excipient interaction on the production of niosomes. *AAPS Pharmaceutical Sciences and Technology*, 13(3): 826-835.
17. Mabboh NA, Donaldson DS, Ohno H, Williams IR, Mahajan A (2013). Microfold (M) cells: immunosurveillance posts in the intestinal epithelium. *Mucosal Immunology*, 6: 666-667.
18. Simecka JW (1998). Mucosal immunity of the gastrointestinal tract and oral tolerance. *Advanced Drug Delivery Reviews*, 34: 235-259.
19. Ghee MC, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H (1997). The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine*, 10: 75-81.
20. Hussain N, Jaitley V, Florence AT (2001). Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics. *Advances in Drug Delivery Reviews*, 509(1-2): 107-42.
21. Coles HE (1986). *Veterinary Clinical Pathology* (4th Edn.). W.B. Saunders Company, U.S.A. pp 279-297.
22. Echeonwu BC, Ngele MB, Echeonwu GON, Joannis TM, Onovoh EM, Paul G (2008). Response of chickens to oral vaccination with Newcastle disease virus vaccine strain I2 coated on maize offal. *African Journal of Biotechnology*, 7(10):1594-1599.