Original Article

An *In Vitro* Comparative Study of Aflatoxin B1 Adsorption by Thai Clay and Commercial Toxin Binders

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Abstract

Twenty samples of Thai clay from fourteen provinces and seven commercial toxin binders were investigated for their adsorption capacity of aflatoxin B1 (AFB_1) *in vitro*. Each sample of 5 mg/l AFB_1 solution was shaken at 25^{0}C for 24 hours and supernatants of centrifuged samples were analyzed for concentrations of AFB_1 using a UV spectrophotometer. Adsorption capacity was calculated and applied to isothermic equations. The results indicated that Thai clays was capable of sequestering AFB_1 from aqueous solution differently and Thai clay from Lopburi and Lamphun provinces had the highest adsorption capacity, similar to commercial binders. S-shaped isothermic curves were observed for all samples having adsorption capacity greater than 4 x 10^{-3} mol/kg. These isothermic data were fitted using a modified Freundlich model that suggested that the samples possessed multilayered or multiple adsorption sites for the toxin. The clay from Lopburi and Lamphun had maximum adsorption capacity (Q_{max}) of 4.76 x 10^{-3} mol/kg and 4.68 x 10^{-3} mol/kg, respectively, whereas the commercial binders had Q_{max} that ranged from 4.38 x 10^{-3} mol/kg to 5.07 x 10^{-3} mol/kg. Inductive couple plasma spectrometry and X-ray diffraction spectrometry of the clay samples demonstrated that the clay from Lopburi and Lamphun contained montmorillonite as a major component, similar to the bentonites. It was concluded that Thai clay from Lopburi and Lamphun provinces could absorb AFB_1 *in vitro* efficiently, similar to commercial toxin binders.

Keywords: adsorption, isotherm, montmorillonite, mycotoxin, Thai clay

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บทคัดย่อ

การศึกษาเปรียบเทียบประสิทธิภาพดินเหนียวในประเทศไทยและสารดูดซับในเชิงพาณิชย์ในการดูดซับสารพิษอะฟลาทอกซินบี 1 ในหลอดทดลอง

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การศึกษานี้ได้นำตัวอย่างดินเหนียวจาก 20 แหล่งใน 14 จังหวัดของประเทศไทยและสารดูดซับสารพิษอะฟลาทอกซินเชิงพาณิชย์ 7 ตัวอย่าง มาศึกษาเปรียบเทียบความสามารถในการดูดซับสารพิษอะฟลาทอกซินบี 1 ในหลอดทดลอง โดยการนำตัวอย่างมาแยกเขย่าในหลอดแก้วที่มีสารละลายสารพิษความเข้มข้น 5 มก./ลิตร ที่อุณหภูมิ 25 องศาเซลเซียส นาน 24 ชั่วโมง หลังจากนั้นทำการปั่นเหวี่ยง และนำสารละลายส่วนบนมาตรวจวัดปริมาณอะฟลาทอกซินด้วยเครื่องยูวีสเปคโตรโฟโตมิเตอร์ ความยาวคลื่น 362 นาโนเมตร ผลการศึกษาแสดงให้เห็นว่าดินเหนียวของไทยจากแต่ละแหล่งสามารถดูดซับสารพิษได้แตกต่างกัน โดยดินเหนียวจากจังหวัดลพบุรีและลำพูนมีประสิทธิภาพในการดูดซับสารพิษสูงกว่าดินจากแหล่งอื่น แต่ไม่แตกต่างจากสารดูดซับในเชิงพาณิชย์ และเมื่อนำข้อมูลการดูดซับสารพิษของสารตัวอย่างที่มีค่าความสามารถในการดูดซับสารพิษมากกว่า 4×10^{-3} โมล/กก. มาศึกษาไอโซเทอร์มพบว่า สร้างกราฟได้เป็นรูปตัวเอส ที่ประยุกต์เข้ากับสมการโมดิฟายฟรุนดิชได้ดี ซึ่งแสดงว่าสารตัวอย่างสามารถดูดซับสารพิษได้แบบหลายตำแหน่งและหลายชั้นในโครงสร้าง นอกจากนี้ยังพบว่าดินเหนียวจากจังหวัดลพบุรีและลำพูนมีความสามารถสูงสุดในการดูดซับสารพิษที่ 4.76×10^{-3} และ 4.68×10^{-3} โมล/กก. ตามลำดับ ส่วนสารดูดซับในเชิงพาณิชย์สามารถดูดซับสารพิษได้ระหว่าง 4.38×10^{-3} ถึง 5.07×10^{-3} โมล/กก. และจากการศึกษาองค์ประกอบและโครงสร้างของตัวอย่างสารดูดซับด้วยอินดักทีฟคัพเปิ้ลพลาสมา สเปคโตรเมทรีและเอ็กซ์เรย์ ดิฟเฟรคชั่น สเปคโตรเมทรีพบว่า ตัวอย่างดินจากจังหวัดลพบุรีและลำพูนมีแร่มอนต์มอริลโลไนท์เป็นองค์ประกอบหลัก เช่นเดียวกับสารดูดซับเบนโทไนท์ในเชิงพาณิชย์ จากการศึกษานี้สรุปได้ว่าดินเหนียวจากจังหวัดลพบุรีและลำพูนมีประสิทธิภาพสูงในการดูดซับสารพิษอะฟลาทอกซินในหลอดทดลอง และไม่แตกต่างจากสารดูดซับในเชิงพาณิชย์

คำสำคัญ: ดินเหนียว สารดูดซับ มอนต์มอริลโลไนท์ สารพิษ ไอโซเทอร์ม

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Introduction

Aflatoxins (AF) are toxic metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B_1 (AFB_1) is widely known as carcinogenic and the most hepatotoxic of natural occurring AF (Hueber et al., 2004; Godfrey et al., 2013). One strategy to detoxify AF is to add toxin binder to animal feed reducing the bioavailability of the toxin absorbed through digestive tract (Basalan et al., 2006). This approach is considered as cost-effective and practical, particularly when using in contaminated feed on an industrial scale (Hueber et al., 2004; Pimpukdee et al., 2004; Kossolova et al., 2009). To date, several *in vitro* studies have shown that binders such as aluminosilicate, bentonite, zeolite, activated charcoal, and chitin-chitosan effectively absorb AFB_1 *in vivo* (Khajarern et al., 2003; Pasha et al., 2007; Manafi, 2011; Khadem et al., 2012; Rao and Chopra, 2012; Sadeghi et al., 2012; Neeff et al., 2013).

Previous studies have applied methods of equilibrium isothermal analysis to characterize the adsorption of AF onto the surfaces of toxin binders (Grant and Phillips, 1998; Pimpukdee et al., 2000). These methods provide evidence of the molecular mechanisms involved with different binders and allow comparisons of similarities and differences. Binders have distinctive molecular structures and bind AFB_1 differently (Phillips, 1999). A comparison of Thai clay with commercial binders for their adsorption capacity of AFB_1 has not been reported. Thus, the main objective of this study was to investigate the adsorption capacity and affinities of Thai clay and compare them to commercial toxin binders using a Langmuir and modified Freundlich isotherm modeling approach.

Materials and Methods

Chemicals: Standard AFB_1 was purchased from Sigma Chemical Co. (St. Louis, USA) and seven commercial binders, three with bentonites (BN) and four without bentonites (NB), were obtained from Thai suppliers. Twenty samples of Thai clay (S) were collected from fourteen provinces as follows: Lopburi (S1), Lamphun (S2), Nakorn Ratchasima (S3-S4), Buriram (S5-S6), Phetchabun (S7), Phitsanulok (S8-S9); Lampang (S10-S11), Nakorn Srithammarat (S12), Kanchanaburi (S13), Ratchburi (S14-S15), Suphanburi (S16), Phetburi (S17-S18), Prachuab Khirikhan (S19) and Chonburi (S20)(Table 1). All clay was sieved to achieve particle sizes less than 60 µm. Highly purified water (18 MΩ•cm) was prepared by processing deionized water through a Milli-Q^{uf+} system.

Adsorption Capacity: After mixing each clay and binder sample into 5 mg/l AFB_1 solutions at 0.25% (w/v) for binder to solution ratio, the mixtures were shaken at 200 x g for 24 hours at 25^0C (Innova 4060 Shaker, New Brunswick Scientific, USA). After shaking, the samples were centrifuged (Beckman Coulter, USA) at 12,000 x g for 30 min, and the supernatants were analyzed to determined the concentrations of AFB_1 using a UV spectrophotometer (Perkin Elmer, USA) at a wavelength of 362 nm (Pimpukdee et al., 2000).

Isothermal Adsorption: Aflatoxin B1 solutions having the concentrations from 0.5 to 8.0 µg/ml were prepared. Isothermic studies utilized 4.0 ml solution of AFB_1 at each concentration, diluted in purified water containing 10.0 mg of each sample. The equilibrating condition and the separation procedure for samples and control tubes were described as above. Supernatants were analyzed further using UV spectrophotometer at wavelength 362 nm (Grant and Phillips, 1998; Pimpukdee et al., 2000). Adsorption capacity was calculated based on the amount of AFB_1 left in the solution (C_e), and the amount of AFB_1 absorbed (q) for each sample. Data were later transferred to a table curve 2D 3V (Jandel Scientific, USA) to fit a Langmuir isothermic model (LIM) or a modified Freundlich isothermal adsorption model (MFM) (Mayura et al., 1998; Pimpukdee et al., 2000).

Analyses of composition and structure: Inductive couple plasma optical emission spectrometry (ICP-OES; Optima 3000, Perkin-Elmer, USA) was used to analyze the composition of the Thai clays and commercial binders having a high absorptive capacity for AFB_1. The samples were further analyzed for their molecular structures using X-ray diffraction spectrometry (XRD; X'Pert MRD, PANalytical, B.V., The Netherlands).

Results

Twenty samples of Thai clay and seven commercial toxin binders were evaluated for their ability to absorb AFB_1. Table 1 shows the mean binding capacity of each commercial binder, which ranged from 4.62 x 10^{-4} to 5.07 x 10^{-3} mol/kg. The NB1 binder provided the highest adsorption capacity of AFB_1 (5.07 x 10^{-3} ± 3.67 x 10^{-5} mol/kg). The BN1-3 had moderate adsorption capacity; 4.74 x 10^{-3} ± 1.50 x 10^{-5}, 4.65 x 10^{-3} ± 2.78 x 10^{-5} and 4.38 x 10^{-3} ± 1.51 x 10^{-5} mol/kg, respectively. For the Thai clay, the highest mean adsorption capacity was found in Lopburi clay (S1) (4.76 x 10^{-3} ± 8.20 x 10^{-5} mol/kg), followed by the clay from Lamphun (S2) (4.68 x 10^{-3} ± 7.41 x 10^{-5} mol/kg)(Table 1). The clay from Nakorn Srithammarat (S12) had the highest distribution constant (6.70 x 10^7) (Table 1).

From the isothermic study, S-shaped isotherms were obtained for samples with the adsorption capacity greater than 4 x 10^{-3} mol/kg. The amount of absorbed toxin increased linearly as the concentration of the AFB_1 increased. Figure 1 shows an example of isothermic plots of three representative groups: bentonite 1 (BN1), non-bentonite binder 1 (NB1), and Thai clay from Lopburi (S1). The equation for LIM ($q = Q_{max} [K_dC_e/(1 + K_dC_e)]$) and the MFM ($q = Q_{max} [K_dC_e]^n$), where q is the amount of AFB_1 adsorbed, Q_{max} is the maximum amount of AFB_1 adsorbed, C_e is the equilibrium concentration of AFB_1 in solution, and K_d is distribution constant (affinity parameter) were selected to fit the data. The LIM is generally applied to the monolayer adsorption, whereas the MFM is appropriate for the multilayer or multiple-site adsorption. With user defined functions, the isothermic data indicated that all absorbents having the adsorption capacity greater than 4 x 10^{-3} mol/kg fitted to the MFM better than the LIM. The MFM had higher correlation coefficients (r^2) for the absorbents in the range of 0.80178-0.98963 and allowed a quantitative comparison of Q_{max} and K_d for absorbents as expressed in Table 1.

The results of the composition and structure analyses demonstrated that BN1 and the Thai clay from Lopburi and Lamphun contained montmorillonite as a major component, having a deviated peak from the beam at angle 2θ approximately 6.4 (Fig 2). It was also noted that Thai clays from these two provinces contained greater percentages of montmorillonite than the other Thai clay.

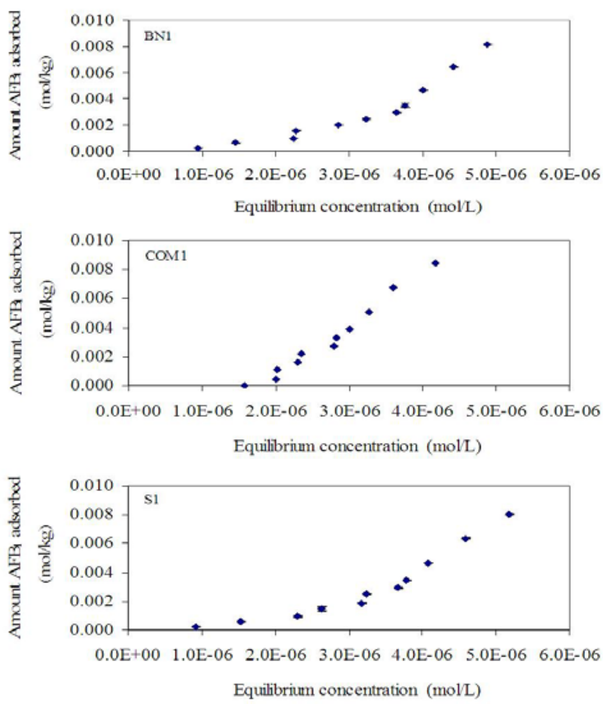

Figure 1 The S-shaped isothermic plots for AFB1 adsorption to bentonite (BN1), non-bentonite clay (NB1) and Thai clay (S1), respectively. The standard deviations on each data point of adsorbed AFB1 were small.

Table 1 Comparison of isotherm fitted parameters of AFB_1 adsorption onto BN, NB an S samples at 25⁰C .

Adsorbent	Langmuir Model(LM)[a]			Modified Freundlich Model (MFM)[b]		
	r^2	Q_{max}	K_d	r^2	Q_{max}	K_d
BN1	0.692	0.993	1.12E+03	0.97903	4.74E-03	2.52E+05
BN2	0.602	0.873	1.24E+03	0.93621	4.65E-03	2.84E+05
BN3	0.848	0.49	1.44E+03	0.88581	4.38E-03	3.60E+05
NB1	0.589	0.817	1.64E+03	0.96648	5.07E-03	4.62E+05
NB2	0.581	0.705	1.71E+03	0.93575	4.85E-03	4.26E+05
NB3	0.614	0.72	1.10E+03	0.98803	4.40E-03	3.04E+05
NB4	0.699	0.673	1.45E+03	0.8976	4.62E-04	4.71E+05
S1	0.69	1.026	1.03E+03	0.98905	4.76E-03	2.37E+05
S2	0.64	1.051	9.42E+02	0.98864	4.68E-03	2.31E+05
S3	0.656	0.892	8.73E+03	0.90105	3.54E-03	3.30E+05
S4	0.963	0.172	3.35E+03	0.96626	1.96E-03	2.76E+05
S5	0.879	0.55	8.56E+02	0.97249	1.66E-03	2.15E+05
S6	0.899	0.731	1.24E+03	0.97004	3.18E-03	2.54E+05
S7	0.917	0.5	1.53E+03	0.96719	1.87E-03	3.32E+05
S8	0.672	1.023	9.82E+02	0.98963	2.95E-03	2.72E+05
S9	0.555	1.092	1.53E+03	0.8645	3.12E-03	4.41E+05
S10	0.746	0.569	1.96E+03	0.84637	1.34E-03	4.79E+05
S11	0.78	0.65	8.94E+02	0.97383	9.61E-04	2.98E+05
S12	0.844	0.666	2.06E+03	0.95424	2.15E-03	6.70E+07
S13	0.943	0.148	2.11E+03	0.94789	7.89E-04	3.54E+05
S14	0.667	0.521	6.99E+02	0.80178	7.10E-04	2.38E+05
S15	0.749	0.62	7.77E+02	0.95252	8.91E-04	3.04E+05
S16	0.743	0.591	2.45E+03	0.88537	2.64E-03	4.21E+05
S17	0.687	0.544	7.12E+02	0.83265	7.5780-04	2.55E+05
S18	0.816	0.657	6.42E+02	0.92454	8.62E-04	4.76E+05
S19	0.938	0.436	1.35E+03	0.98369	1.64E-03	2.90E+05
S20	0.91	0.419	1.87E+03	0.9642	4.55E-04	3.19E+05

[a)] $q = Q_{max}[K_dC_e/(1+ K_dC_e)]$, [b)] $q = Q_{max}[K_dC_e]^n$ where q: absorbed amount (mol/kg), K_d : distribution coefficient, C_e : equilibrium concentration (mol/l), n : heterogeneity factor, BN : bentonite, NB : non-bentonite toxin binder, S : Thai clay

Discussion

This isothermic adsorption study of AFB_1 Thai clay from different provinces and commercial binders was performed at 25⁰C to assess potential use in the agricultural industry. S-shaped isotherms were observed for samples with adsorption capacity greater than 4 x 10^{-3} mol/kg. (Fig 1). Generally, the S isotherm is observed when a molecule does not have a strong affinity for the surface, until there is a significant amount absorbed, and the slope increases as the affinity for the surface increases (Grant and Phillips, 1998; Hinz, 2001). This occurs because the solute molecule has modified the surface or has begun to bind to the previously adsorbed molecules. The S-type curve usually appears when three conditions are fulfilled: the solute molecule (1) is mono functional, (2) has moderate intermolecular attraction, causing it to pack vertically on the absorbing layer, and (3) meets strong competition for substrate sites from molecules of the solvent or of another absorbing species.

All samples having the adsorption capacity greater than 4 x 10^{-3} mol/kg fitted to the MFM better than the LIM. This implies that the binders possess multiple-sites or have multilayer adsorption (Hinz, 2001; Pimpukdee et al., 2004). The fitting result for MFM indicated that the clay from Lopburi and Lamphun had relatively high average maximum adsorption capacity and were not different from other commercial toxin binders. Therefore, it was concluded that the clay from Lopburi and Lamphun was capable of absorbing AFB_1 efficiently. Adsorption of AFB_1 can occur on external surfaces, interlayer surfaces, original

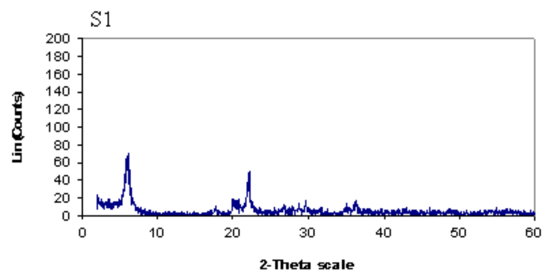

Figure 2 The X-ray diffraction spectrogram of Thai clay from Lopburi (S1) for the adsorption of AFB_1.

edge sites, interlayer exchangeable cations, or on previously absorbed molecules (Hinz, 2001; Diaz et al., 2002; Hueber et al., 2004). Previous studies have shown that montmorillonite has a high Q_{max} for toxin, because of the extremely large and highly selectivity specific surface area, high cation exchange capacity (CEC), high cohesion and adhesion, and high equilibrium capacity (Pimpukdee et al., 2000, 2004). In contrast, some other clay (pyrophyllite, kaolinite, illite, chlorite, talc, mica) has relatively low Q_{max} due to low CEC and relatively small internal surface areas (Pimpukdee et al., 2000).

In addition, the results of ICP-OES and XRD analyses demonstrated that the Thai clay collected from Lopburi and Lamphun contained montmorillonite as a major component and its binding capacity was similar to bentonite binders. Therefore, the composition and structure of the Thai clay from these two provinces were comparable in efficiency to commercial bentonites for binding the toxin.

In conclusion, Thai clay from Lopburi and Lamphun can absorb AFB_1 *in vitro* similar to other commercial toxin binders.

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References

Basalan M, Gungor T, Aydogan I, Hismiogullari SE, Erat S and Erdem E 2006. Effects of feeding mycotoxin binder (HSCAS) at later ages on gastrointestinal environment and metabolism in broilers. Archiva Zootechinca. 9(1): 5-9.

Diaz DE, Hagler WM, Hopkins BA and Whitlow LW 2002. Aflatoxin binders I: *In vitro* binding assay for aflatoxin B1 by several potential sequestering agents. Mycopathol. 156(3): 223-226.

Godfrey SB, David K, Lubega A, Ogwal-Okeng J, Anokbonggo WW and. Kyegombe DB 2013. Review of the biological and health effects of aflatoxins on body organs and body systems. In: Aflatoxins-Recent Advances and Future Prospects. MR Abyaneh (ed). Croatia: Intech. 239-265.

Grant PG and Phillips TD 1998. Isothermal adsorption of aflatoxin B1 on HSCAS clay. J Agri Food Chem. 46(2): 599-605.

Hinz C 2001. Description of sorption data with isotherm equations. Geoderma. 99(4): 225-243.

Huebner HJ, Herrera P and Phillips TD 2004. Clay-based interventions for the control of chemical and microbial hazards in food and water. In: Preharvest and Postharvest Food Safety-Contemporary Issues and Future Directions. RC Beier, SD Pilai and TD Phillips (eds). Iowa, USA: IFT Press and Blackwell Publishing. 389-402.

Khadem AA, Sharift SD, Barati M and Borji M 2012. Evaluation of the effectiveness of yeast, zeolite and active charcoal as aflatoxin absorbents in broiler diets. Global Veterinaria. 8(4): 426-432.

Khajarern J, Khaharern S, Moon TH and Lee JH 2003. Effects of dietary supplementation fermented chitin-chitosan (Fermkito) on toxicity of mycotoxin in ducks. Asian-Aust J Anim Sci. 16(5): 706-713.

Kossolova A, Stroka J, Breidbach A, Kroeger K, Ambrosio M, Bouten K and Ulberth F 2009. Evaluation of the effect of mycotoxin binders in animal feed on the analytical performance of standardized methods for the determination of mycotoxins in feed. JRC Scientific and Technical Reports. The European Commission 5-12.

Manafi M 2011. Evaluation of different mycotoxin binders on aflatoxin B1 (*Aspergillus parasiticus*) produced on rice (*Oriza sativa*) on fertility, hatchability, embryonic, mortality, residues in egg and semen quality. Adv Environ Biol. 5(13): 3818-3825.

Mayura K, Abdel-Wahab MA, Mckenzi KS, Sarr BA, Edwards JF, Naguib K and Phillips TD 1998. Prevention of maternal and developmental toxicity in rats via dietary inclusion of common aflatoxin sorbents: Potential for hidden risks. Toxicol. Sci. 41(2): 175-182.

Neeff DV, Ledoux DR, Rottinghaus GE, Bermudez AJ, Dakovic A, Murarolli RA and Oliveira CAF 2013. *In vitro* and *in vivo* efficacy of a hydrated sodium calcium aluminosilicate to bind and reduce aflatoxin residues in tissues of broiler chicks fed aflatoxin B1. Poult Sci. 92(1): 131-137.

Pasha TN, Farooq MU, Khattak FM, Jabbar MA and Khan AD 2007. Effectiveness of sodium bentonite and two commercial products as aflatoxin absorbents in diets for broiler chickens. Anim Feed Sci Tech. 132(1): 103-110.

Phillips TD 1999. Dietary clay in the chemoprevention of aflatoxin-induced disease. Toxicol. Sci. 52(1): 118-126.

Pimpukdee K, Ake C, Lemke SL, Mayura K and Phillips TD 2000. High affinity sorption of aflatoxin B_1 by hectorite clay. Toxicol Sci. 54(1): 143.

Pimpukdee K, Kubena LF, Bailey CA, Huebner HJ, Afriyie-Gyawu E and Phillips TD 2004. Aflatoxin induced toxicities and depletion of hepatic vitamin A in young broiler chicks: Protection of chicks in the presence of low levels of novasil plus in the diet. Poultry Sci. 83(5): 737-744.

Rao SBN and Chopra RC 2012. Influence of sodium bentonite and activated charcoal on aflatoxin M1 excretion in milk of goats. Small Rumi Res. 41(3): 203-213.

Sadeghi AA, Ahmadi-Mazhin H, Shawrang P and Mohammadi-Sangcheshmeh A. 2012. Effects of MOS and heat activated sodium bentonite as aflatoxin absorbents on antibody titers against Newcastle disease and infectious bursal disease viruses in broiler chickens. World Appli Sci J. 18(1): 127-129.