

An in-vitro evaluation of the capacity of local Tanzanian crude clay and ash based materials in binding aflatoxins in solution

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Abstract

Aflatoxins in feeds cause great health hazards to animals and in advance, to human. Potential of crude clays designated AC, KC, CC and MC and ashes VA and RA were evaluated for their capacity to adsorb aflatoxins B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁) and G₂ (AFG₂) relative to a commercial binder Mycobinder^R (Evonik Industries AG) using in-vitro technique. On average, CC, VA, KC, MC, AC, RA and Mycobind^R adsorbed 39.9%, 51.3%, 61.5%, 62.0%, 72.6%, 84.7% and 98.1% of the total aflatoxins in buffered solution, respectively. The capacity of AC and RA was statistically (p<0.05) better in binding aflatoxins next to Mycobind^R. Capacity of the TBM and Mycobind^R to bind aflatoxins, seemed to follow the trend of their cation exchange capacity (CEC). The CEC (meq/100g) of CC, MC, KC, VA, AC, RA and Mycobind^R were 7.0, 15.4, 18.8, 25.4, 27.2, 27.2 and 38.9, respectively. On average 96.3%, 42.7%, 80.8% and 32.1% of AFB₁, AFB₂, AFG₁ and AFG₂ were adsorbed, respectively. Binding capacity of the clays and ashes relative to Mycobind^R was about 100% for AC and RA, 50% for KC, MC and VA and 33.3% for CC. The AC and RA seem to be promising resources in binding aflatoxins in solution.

Keywords: Clays, Ashes Aflatoxins, Binding capacity, In-vitro and Contaminated feeds

1.0 Introduction

Aflatoxins are natural toxins produced in foods and feeds, primarily, by certain species of fungi, specifically *Aspergillus flavus* and *Aspergillus parasiticus*, when conditions are favourable for fungal growth and subsequent toxin formation. Aflatoxins exist in four forms of health, agricultural and economic importance, namely aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁) and G₂ (AFG₂) (1, 2, 3). The most toxic and abundant aflatoxins is AFB₁ (4). Almost all feed resources contain certain levels of naturally occurring aflatoxins and any level of dietary aflatoxins poses a certain level of health risk (5). Studies show that aflatoxins in feeds depress growth and production performance of animals (6, 7). When animals are fed naturally aflatoxin-contaminated feeds, the toxins (mostly AFB₁) are secreted in cow milk or retained in hen eggs as aflatoxin M₁ (AFM₁) (8, 9, 10).

It is therefore imperative to prevent and reduce hazards of aflatoxin contamination of feeds for protection and promotion of human and animal health. Some of the techniques used to reduce aflatoxin contamination of feeds are thermal inactivation and irradiation as physical techniques

and treatment of the feeds with acidic or alkaline solutions, ozone treatment and ammoniation as chemical techniques and detoxification by microbial agents as biological techniques (11, 12). These techniques are mostly applied in the animal industry and are reported to have some limitations including costs implications, requirement of some complicated facilities, reduction of dietary palatability and nutritional values, also creating danger of unsafe chemical residual (13). Techniques involving toxin binders (also called adsorbents or sequesters) have been employed owing to their economic feasibility, applicability and nutritional safety.

Many types of crude or refined materials including clays, cellulose products, yeast cell wall products and activated charcoal products are envisaged to have ability to sequester or bind aflatoxins (14, 15, 16). The potential binding capacity of these materials are known to vary depending on their nature and source (17). According to Kannewischer *et al.* (18); Vekiru *et al.*, 2007(cited by Vekiru *et al.* (17) there is no existing clear generic linear relationship between binding effectiveness and specific adsorbent properties, such as elemental and mineralogical content, cation exchange capacity (CEC) and pH levels of materials. The binding potential of some materials particularly clays seems to be the function of their chemical composition, such as Ca^+ and K^+ ions present in the framework configured by Silicon, Aluminium and Iron oxide. Studies show that Alumino-silicates have wide variation of these elements (Table 1). In South American countries, ashes such as soda ash and wood ash have been used in some food processes such as in niztamalization for corn tenderization where dietary aflatoxins load is also reduced owing to breakage of aflatoxin lactone-ring by the ash alkalinity (19). In Tanzania farmers are using an imported binder that has proven to be useful in terms of protecting livestock from aflatoxin exposure. However, the imported binders are expensive; the high cost of importing these products which are clay-based materials can be avoided if local resources and sources with similar potential are identified. Our experience in animal husbandry in Tanzania shows that there is a number of clay and ash based materials directly eaten by human or added to feeds and foods for various purposes.

Table 1: Percent structural components in clay samples and Rice-husk ashes collected from various locations

# of samples	Mean/Rang e	Percent structural components of clays and RHA samples					Source
		SiO_2	Al_2O_3	Fe_2O_3	CaO	K_2O	
11 clays	Mean	59.6	19.0	5.2	1.7	0.8	(20)
	Range	1.1-69.0	0.5-21.7	0.2-14.8	0.1-6.8	0.1-3.3	
10 clays	Mean	55.3	13.7	4.4	1.4	1.3	(21)
	Range	44.3-71.0	8.4-20.1	1.4-8.0	0.1-2.4	0.1-2.6	
		80.2	13.2	2.7	0.2	0.1	(22)
RHA	Mean	88.3	0.5	0.7	0.7	2.9	(23)
RHA	Mean	89.0	1.2	1.3	1.0	1.2	(24)
RHA	Mean	93.4	0.1	0.1	0.3	1.4	(25)

RHA: Rice-husk ash

The clay based materials are sold for geophagia purposes, mostly demanded by some groups of women especially pregnant ones. Ashes have been used in traditional cookery of some local foods such as corn recipes and in feeds as ration improvers or appetisers.

We hypothesize that these materials could provide aflatoxin binding capacity equivalent to the imported product. Arbitrarily, we selected clays obtained in the regions of Arusha, Kilimanjaro, Morogoro and Coast and also volcanic ash and rice-husk ash from Arusha region. The potential of these materials in binding toxins has been speculated from the instinct of among animals, birds and human eating soils, which shield them from toxic effects of some ingested natural toxins (26, 27).

The objective of the study was therefore to evaluate the chemical composition and the capacities of the above mentioned materials in binding aflatoxins.

2.0 Results

2.1 Chemical composition of the TBM and Mycobind^R

The major minerals contained in the TBM and Mycobind^R are presented in Table 2. Muscovite minerals was observed in AC and KC, Kaolinite in CC and MC, Leucite in MC, Microline and Ephicite in VA, Albite and Terranovaite in RA, Metanatrolite and Phlogopite in MycobindR.

Table 2: Mineralogical and chemical composition of the TBM and Mycobind^R

TBM and Mycobind ^R ID	Prominent Minerals	Chemical formula
AC	Muscovite	$KAl_2(AlSi_3O_{10})(F,OH)_2$
	Hematite-proto	$Fe_{1.9}H_{0.06}O_3$
KC	Quartz	SiO_2
	Muscovite	$KAl_2(AlSi_3O_{10})(F,OH)_2$
CC	Lizardite	$Mg_3Si_2O_5(OH)_4$
	Kaolinite	$Al_2Si_2O_5(OH)_4$
MC	Kaolinite	$Al_2Si_2O_5(OH)_4$
	Leucite	$K[AlSi_2O_6]$
VA	Lizardite	$Mg_3Si_2O_5(OH)_4$
	Pigeonite	$(Ca, Mg, Fe) (Mg, Fe)Si_2O_6$
RA	Microcline	$KAlSi_3O_8$
	Ephesite	$NaLiAl_2(Al_2Si_2)O_{10}(OH)_2$
MycobindR	Albite	$NaAlSi_3O_8$ or $Na_{1.0-0.9}Ca_{0.0}$
	Terranovaite	$NaCaAl_3Si_{17}O_{40}8H_2O$
MycobindR	Sepiolite	$Mg_4Si_6O_{15}(OH)_2 \cdot 6H_2O$
	Metanatrolite	$Na_2Al_2Si_3O_{10}$
	Phlogopite	$KMg_3(AlSi_3O_{10})(F,OH)_2$
	Andradite /Melanite	$Ca_3Fe_2(SiO_4)_3$

The elemental-oxide composition of the TBM and Mycobind^R is shown in Table 5. All samples of the TBM and Mycobind^R contained Aluminium and Silicon elements as backbone of the minerals. Other important elements observed as parts of the chemical formula of the prominent minerals in the TBM and Mycobind^R were Iron in AC, VA and MycobindR, Calcium in VA, RA and MycobindR, Potassium in all materials except CC and RA. The VA and Mycobind^R had minerals containing all the main elements Aluminium, Silicon, Iron, Calcium and Potassium.

RA showed the lowest content of Aluminium oxide (alumina) of 0.5%, all the other TBM had content above that of Mycobind^R at 5.1%. Percent Silicon oxide (Silica) contents in CC and RA were above that of Mycobind^R while the TBM had contents from 22-32.8%; a level lower than that of Mycobind^R (49%). The VA and RA had percent contents of Potassium oxide a little bit higher than that of Mycobind^R. The VA had Calcium oxide a bit higher than that of Mycobind^R while the rest of the TBM had percent contents below that of Mycobind^R. The AC and RA had the highest and the lowest contents of Iron oxide, respectively. Except RA and CC which had lower percent of iron oxide contents, the AC, KC, MC and VA had values above that of Mycobind^R.

The values of CEC for the TBM are also shown in Table 05. The values of CEC for the TBM ranged from 7 meq/100g for CC to 27.2 meq/100g for RA. All the TBM had lower values of CEC compared to that of Mycobind^R (38.9 meq/100g).

CEC, Cation exchange capacity

2.2 Capacity of the binders to bind aflatoxin

Percent aflatoxin binding capacity for the TBM are presented in Table 3 (across the columns). The percent binding capacity of the TBM ranged from a minimum value of 40 for CC to a maximum value of 85 for RA relative to 98 for the Mycobind^R. The mean proportions of aflatoxins as adsorbed by the TBM and MycobindR are also shown in Table 6 (across the rows). Proportions of aflatoxins adsorbed were relatively high for AFB1 and AFG1 and low for AFG2 and AFB2.

Table 3: *In-vitro* binding capacity of various clay and ash based materials for aflatoxins

TBM identity	Mean percent of bound individual aflatoxin				Mean percent of total aflatoxin bound	SEM
	AFB1	AFB2	AFG1	AFG2		
AC	97.9	60.6	99.9	32.2	72.6 ^{ab}	32.5
KC	95.4	40.1	96.1	14.5	61.5 ^{bc}	40.9
CC	96.6	14.4	31.3	17.3	39.9 ^c	38.5
MC	95.6	32.6	94.6	25.3	62.0 ^{bc}	38.3
VA	97.9	28.9	71.5	30.7	57.3 ^{bc}	33.5
RA	94.6	79.8	91.5	72.7	84.7 ^{ab}	10.2
Mycobind ^R	97.7	99.2	98.8	96.4	98.1 ^a	1.3
Mean	96.5 ^a	50.8 ^b	83.4 ^a	41.3 ^b		
SEM	1.4	30.4	24.9	31.0		

SEM = Standard error of the means; Means with similar superscripts do not differ significantly

The relationship between CEC values of TBM and their elemental-oxides concentration is shown in Figure 1. The relationship presented as correlation coefficients was positive and relatively higher with CaO (0.6), K2O (0.6) and Fe2O3 (0.1) and negative with SiO2 (-0.1) and Al2O3 (-0.9). Similarly, the relationship between percent binding capacity of the TBM and their chemical properties is presented in Figure 2. Their relationship presented as correlation coefficients was positive and relatively higher with values of CEC (0.9), K2O (0.5), CaO (0.3) and SiO2 (0.2) and negative with Fe2O3 (<-0.1) and Al2O (-0.9).

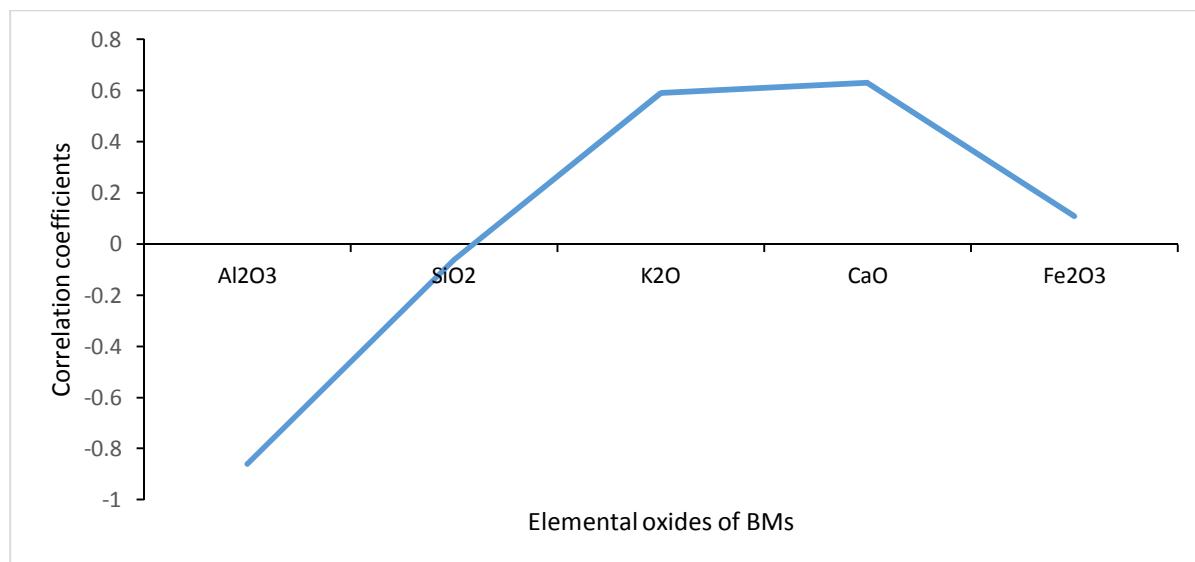


Figure 1: Relationship between values of CEC and the elemental-oxide concentration in TBM

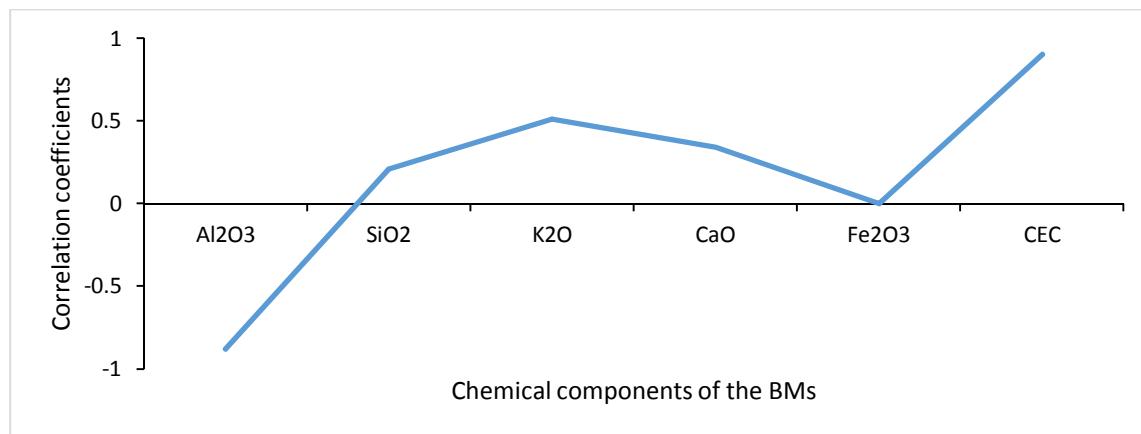
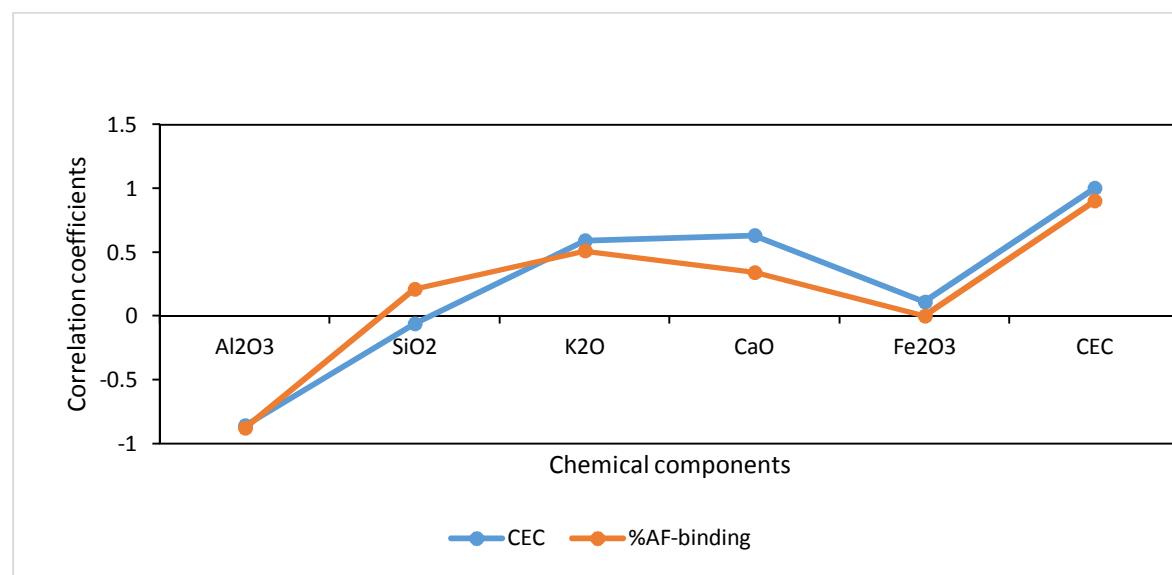


Figure 2: Relationship between percent binding capacity of TBM and their chemical components

Relationship between the binding capacity and CEC of the TBM with respect to their chemical factors is shown in Figure 3. The relationship presented as correlation coefficient was as high as 0.9.

Table 4: The major elemental-oxides composition of the TBM and Mycobind^R

TBM/Mycobind ^R	Elemental-oxide composition of the TBM and MycobindR (%)					CEC (meq/100g)
	Al ₂ O ₃	SiO ₂	K ₂ O	CaO	Fe ₂ O ₃	
AC	18.0	26.0	0.22	0.79	45.31	27.2
KC	25.0	31.0	0.01	0.24	39.73	18.8
CC	32.8	61.3	0.63	0.49	2.14	7.0
MC	24.0	34.8	0.52	0.54	36.1	15.4
VA	15.0	22.0	8.78	14.9	26.2	25.4
RA	0.5	75.7	9.54	1.71	0.59	27.2
Mycobind ^R	5.1	49.0	6.99	13.4	19.8	38.9

**Figure 3: Relationship between the binding capacity, CEC and the concentrations of chemical factors in TBM**

The equivalence of Mycobind^R in binding the total aflatoxins relative to the TBM is shown in Table 5. The binding capacity ratio of Mycobind^R to AC and RA was 1, Mycobind^R to KC, MC and VA was 2 and Mycobind^R to CC was 3.

Table 5: Aflatoxin binding capacity ratio of Mycobind^R to that of the TBM

Aflatoxins	AC	KC	CC	MC	VA	RA
AFB1	1.0	1.0	1.0	1.0	1.0	1.0
AFG1	1.0	1.0	3.2	1.0	1.4	1.1
AFB2	1.6	2.5	6.9	3.0	3.4	1.2
AFG2	3.0	6.6	5.6	3.8	3.1	1.3
Overall	1.0	2.0	3.0	2.0	2.0	1.0

3.0 Discussion

Among the evaluated binding materials, RA and AC had binding capacity almost equal to that of the reference binder, particularly in binding AFB1 and AFG1, which are the most toxic aflatoxins. Possibly the excellent binding power of these materials was due to their relatively high CEC values. The CEC values of both RA and AC were 27.2meq/100g of the materials and are equivalent to the CEC value for the reference material. High CEC values of many binding materials have been reported to promote their capacity to bind aflatoxins (17). The relatively high values of Calcium (Ca^{2+}) and Potassium (K^+) contents in the alumino-silicate minerals of the evaluated materials seemed to promote values of CEC of the materials. Studies have shown that concentrations of Ca^{2+} and K^+ ions make a great contribution to CEC levels in alumino-silicate materials (28). Presence of Silicon (Si^{4+}), Aluminium (Al^{3+}) and Iron (Fe^{3+}) seemed to have low or negative influence on the CEC values of the TBM/Mycobind^R. According to 29, values of CEC increase with decreasing acidity and vice versa. The ions Si^{4+} , Al^{3+} and Fe^{3+} are acidity promoter unlike Ca^{2+} and K^+ (29), hence negatively influencing CEC values of the TBM/Mycobind^R and subsequently their capacity to bind aflatoxins in solution. Disregarding other factors such as structural effect of a material, probably materials like CC showed low capacity for aflatoxins binding partly due to its higher concentration of Al^{3+} and Si^{4+} , and partly due to its relatively higher content of Kaolinite type of mineral, which has low CEC (30). Furthermore, KC and MC could not bind aflatoxins efficiently possibly due to relatively higher concentration of Al^{3+} and Fe^{3+} .

The X-RD analysis showed presence in the TBM, of prominent mineral components that can influence aflatoxin binding. The results showed that just like the Mycobind^R, RA and AC contained major minerals such as Andranite/Melanite, Terranovite and Albite; all of which contain Calcium and Phlogopite as well as Muscovite which contain Potassium. Possibly these components rendered RA and AC relatively superior to others in binding aflatoxins. In aflatoxin binding ions, Ca^{2+} in particular, synchronously bonds to two aflatoxin carbonyls and at the same time binds to the four oxygen atoms of the Si-O ring on the clay binder surface (31). However, AC had low Ca^{2+} and K^+ cations yet its CEC value was relatively high enough to favour high aflatoxin binding capacity. Seemingly, the way active cations such as Calcium and Potassium are incorporated in different structures of the TBM and their associations with other structural elements may affect adsorptive potential of the TBM.

Generally, the chemical composition values for the TBM evaluated in this study were within value ranges reported for alumino-silicate based materials studied for various purposes including as feed additives. The general alumina content in the materials was within the range reported in other studies of 0.45- 21.7% (20) and 13.2% (Massinga *et al.*, 2010) cited by 22 except for CC which contained higher level of alumina at about 33%. Except for RA which showed much higher percent content of silica, the other TBM had content similar to the reported values for clay materials ranging from 1.1-69.0% (mean of 59.6%) (20) and 44.3-71.0% (mean of 55.3%) (20) Just like Mycobind^R, VA and RA had content of Potassium oxide above the previously reported range of 0.1-3.3% (19) and 0.1-2.6% (20) and 0.1% (Massinga *et al.*, 2010) cited by 21 for high aflatoxin binding. Content of Potassium oxide of 0.01% in KC was below the reported levels. Contents of Calcium oxide in all TBM were found within the previously reported range of 0.1-31.4% (19, 20 and Massinga *et al.*, 2010) cited by 21 for binders. Except the CC and RA, the rest of the TBM showed content of Iron oxide above the previously reported range of 0.2-14.8% (19, 20 and Massinga *et al.*, (2010) cited by 21) for binders. From the comparative composition of the TBM it seems that the materials do not differ from other materials of alumino-silicate nature including those proved to bind aflatoxins.

Alumino-silicates based materials have been reported to have CEC (meq/100g) values ranging from 10 (Kaolinite mineral) to 100 (Illite and Smectite minerals) and medium values are found around value of 25 (30). Except for the CC that showed low value of 7 meq/100g, the rest of the

TBM had CEC values within the documented range as were observed from 15.4meq/100g (MC) to 38.9meq/100g for MycobindR.

The results for aflatoxin binding capacity of the TBM concurred with results of other previous related in-vitro studies in which binding capacity levels of clay-based binders such as bentonites (about 90%) has been reported (32, 33). The MycobindR employed as a reference in this study missed manufacturer's information displaying its capacity to bind aflatoxins. However, in our analysis we found that it could bind about 98% of the total aflatoxins subjected to it. A similar product Agrolite-MycobindR evaluated in Kenya showed aflatoxin binding capacity of 95% (34). Regarding minimum experimental set-up standards as suggested by 35, though slightly higher, the binding capacity of 98% observed for the MycobindR in this study matched closely to 95% value reported for the Agrolite-MycobindR.

The binding capacity ratio of MycobindR to TBM observed in this study, conversely implied that AC and RA bind 100%, KC, MC and VA bind 50% and CC binds 33.3% of the total aflatoxins in solution. This indicates though in varying levels, the locally available crude materials (clay and ash based resources) have potential to adsorb aflatoxins in solution media and possibly can reduce aflatoxin contamination of feeds.

The AFB1 and AFG1 were highly adsorbed into the TBM as compared to AFB2 and AFG2. Probably this is because compared to AFB2 and AFG2, the AFB1 and AFG1 have higher polarity of the β -dicarbonyl group which is a key functional group of the aflatoxins (14). With the polarity respect, AFB1 was rendered the most adsorbed by the TBM followed by AFG1. This is advantageous since the toxicity of the aflatoxins tends to follow this order of reactivity, which was also obeyed by our results in this study. The aflatoxin binding capacity of the evaluated materials (especially RA and AC) can be confirmed on *in-vivo* test where the dietary and animal's GIT factors are automatically accommodated. However, since exported binders are costly to farmers in low income countries, occasionally the material can be used in feeds to reduce hazard effects of aflatoxins to animals. In addition, traditionally farmers have been using an array of such materials for various intentions including uses in animal feeds. It has been observed that wild animals and birds are less affected by many natural toxins probably including aflatoxins due to their instincts related to geophagia (26, 27). Harnessing this natural phenomenon may be economically helpful to poor farmers as one of strategies in lowering aflatoxin menace which is difficult to avoid in feeds.

4.0 Conclusion

The test materials we evaluated in the study had varying capacity levels of binding aflatoxins in solution. The crude materials AC (Arusha clay) and RA (Rice-husk ash) have the relatively higher potential to bind aflatoxins equivalent to the commercial product MycobindR employed in the study for reference purpose. Since traditionally these cheap materials are used for various purposes in animals, occasionally they could be utilized to minimize exposure of aflatoxin load to animals through contaminated feeds. Further studies are recommended to test binding capacity of these materials in refined form and when used in combinations of two or more of them, using both on in-vitro and in-vivo trials.

5.0 Materials and methods

5.1 Materials

5.1.1 Test binding materials and their sources

Six crude test binding materials (TBM) were evaluated against a commercial binder, MycobindR as reference material. Of these TBM, four were clays, designated AC, KC, CC and MC and two ash-based materials were volcanic ash (VA) and rice-husk ash (RA). Nature, source and ethno-utilization of the TBM are shown in Table 6. Samples of AC, KC, CC, and MC were obtained by taking several aliquots of a material from various parts of the source or sampling lot making representative samples of about 5kg of each TBM. The samples were taken to laboratory at

Geological Survey of Tanzania for cleaning, grinding, sieving, homogenization and then packed in zip bags for subsequent chemical analyses and evaluation for adsorption capacity.

Table 6: Physical appearance, sources and current uses of the test materials

Material ID	Physical appearance	Source region	Ethno-utilization
Clays			
AC	Brick-red clogs	Arusha	Treatment of human skin infection and ailments
KC	Brownish-red blocks	Kilimanjaro	Geophagial satisfaction
CC	Shiny white granules	Coast	Stomach ailment treatment and for decorations
MC	Brownish-red granules	Morogoro	Geophagial satisfaction
Ashes			
VA	Greyish Volcanic powder	Arusha	Food seasoning and tenderization in traditional cookery, feed additive
RA	Greyish-white fine powder	Various places	Soil fertility improvement

In this and subsequent tables or figures: AC; Arusha clay, KC; Kilimanjaro clay, CC; Coast clay, MC; Morogoro clay, VA; volcanic ash, RA; rice-husk ash

Five kilogram of VA was purchased from the market and the site of production was followed to ascertain its originality, then handled like for the clay samples in the laboratory. Representative sample of rice-husks was taken from rice-millers and incinerated in the laboratory furnace at the Nelson Mandela African Institution of Science and Technology (NM-AIST) a temperature of 550 °C for four hours and make about 5kg of rice-husk ash.

5.1.2 The reference binder

For comparison of the binding capacity of the crude clays and ashes, a commercial mycotoxin detoxifier named Mycobind® (Evonik Industries AG) was purchased from Farmers Centre Limited in Dar es Salaam, Tanzania and employed in the study.

5.1.3 Aflatoxin solution

The stock solution of aflatoxins produced by Romer Labs, Inc. USA was donated by Tanzania Food and Drugs Authority (TFDA).

5.2 Chemical analysis of the test materials

Samples of the TBM and Mycobind® were further homogenized, ground and sieved through 1mm sieve for the subsequent analyses of mineralogical composition, elemental content and cation exchange capacity (CEC).

5.2.1 Mineralogical composition

Samples of the TBM and Mycobind® were analysed for mineralogical composition using non-destructive techniques that employed X-RD analyser (BTX SN 231, Olympus Corporation, Tokyo Japan), a self-calibrated diffractometer depending on temperature. The samples were analysed at a temperature of -45°C. About 15mg of finely ground sample was sieved through 150µm sieve and loaded in the vibrating sample holder of the X-RD analyser for scanning. The results were XRD-spectrum patterns, received on a screen of a computer connected to the analyser showing peaks corresponding to each specific mineral present in the sample.

5.2.2 Elemental-oxide composition

The oxides in the TBM and Mycobind^R were quantified by Minipal-4 a high performance bench top energy dispersive X-ray fluorescence spectrometer (PANalytical MINIPAL-4, EDXRF Spectrometer, The Netherlands). The sample was ground into a fine powder, then about 50g of it was scanned by the spectrometer for metallic oxide composition at an energy dispersion of 30keV. The percent composition of the metallic oxides in each sample was recorded.

5.2.3 Determination of cation exchange capacity (CEC)

The CEC was determined by wet analysis employing Ammonium Replacement Method (Buchner funnels vacuum flasks) as explained by Brady and Weil (35) and involving leaching of exchangeable cations in the TBM/Mycobind^R with ammonium acetate salt solution. The excess salt was removed by ethanol followed by potassium chloride to leach NH₄⁺ which initially replaced other various cations of the TBM/Mycobind^R. The amount of NH₄⁺ released and washed into a beaker beneath Buchner funnels was determined using Kjeldahl distillation method (36) and CEC (meg/100g) of TBM and Mycobind^R was computed using equation (1).

$$\text{CEC} = (\text{mg L}^{-1} \text{ of NH}_4\text{-N in leachate}) \times (0.25/14) \times (100/\text{sample weight (g)}) \text{ mg L}^{-1} \text{ NH}_4\text{-N.} \quad (1)$$

5.3 Experimental design and treatments

5.3.1 Experimental design

The six TBM and Mycobind^R engaged to bind aflatoxins formed seven treatments of the in-vitro experiment. Each of the treatments was replicated into three units (test-tubes).

5.3.2 Preparation of the experimental solutions

The experiment was based on a buffer solution with or without a TBM/Mycobind^R and spiked or non-spiked with aflatoxin solution.

a. Buffer solution

The buffer solution was prepared from Potassium Chloride, Potassium dihydrogen phosphate anhydrous disodium hydrogen phosphate and Sodium chloride in distilled water

b. Diluted aflatoxin solution

The standard solution of combined aflatoxins AFB1, AFB2, AFG1 and AFG2 (250ng/ml) in acetonitrile was diluted to 20ng/ml using distilled water in an amber flask.

c. Solutions of TBM/Mycobind^R and controls

The test solutions contained components as shown below and summarised in Table 7

- (i) Spiked TBM/Mycobind^R: suspension of 0.25% of TBM/Mycobind^R in the buffer solution spiked with 5ml of diluted solution of aflatoxins
- (ii) Non-spiked TBM/Mycobind^R: a control for each binding material containing suspension of 0.25% of TBM/Mycobind^R in the buffer solution.
- (iii) Positive control: the buffer solution spiked with 5ml of diluted solution of aflatoxins and
- (iv) Negative control: the buffer solution only.

For each solution three replications were taken in separate test-tubes as experimental units.

Table 7: Experimental solutions

Solution samples	Composition	# of samples	Replications	Total # of units (tubes)
Spiked TBM/Mycobind ^R	Buffer solution, TBM/Mycobind ^R and diluted aflatoxin solution	7	3	21
Controls				
Non-spiked TBM/Mycobind ^R	TBM/Mycobind ^R and buffer solution	7	3	21
Positive control	Buffer solution and diluted aflatoxin solution	1	3	3
Negative control	Buffer solution	1	3	3

2.3.3 Procedure for *in-vitro* experiment

The *in-vitro* procedure was adopted from Kong *et al.* (36) simulating gastrointestinal pH condition of pigs, representing monogastric animals, which are more prone to aflatoxicosis. A sample of TBM/Mycobind^R was prepared by weighing 0.025g into 10ml of phosphate buffer solution (0.1 M, pH 6.0) making a suspension of 0.25%. An aliquot of 2.5 ml suspension was pipetted into 25 ml centrifuge-tube then 5 ml of the diluted aflatoxin solution was added. Parallel with the TBM/Mycobind^R test treatments, their respective negative controls (non-spiked with the diluted aflatoxin solution) were ran. General positive and negative controls were included to eliminate probable error effects such as due to aflatoxin impurities in the measuring/analysis system hardware and reagents. The positive control contained 2.5ml of phosphate buffer and 5ml of the diluted aflatoxin solution while the negative control contained 5 ml of phosphate buffer solution only. Each solution sample was replicated thrice and pH in each centrifuge-tube was adjusted to 2.0 by adding 1M HCl to simulate pH in the stomach of pigs.

5.3.4 Incubation of the solution samples

All samples were incubated at 39°C in a shaking water bath for two hours and then 1ml of phosphate buffer (0.2 M, pH 6.8) was added to each tube. To simulate the conditions in the small intestine of pigs, pH in all tubes was raised to 6.8 by adding 1M NaOH followed by second phase incubation at 39°C for four hours. After incubation, the mixture was centrifuged and the supernatant was obtained for analysis of residual (unbound) aflatoxins B1, B2, G1 and G2 using High Performance Liquid Chromatography (HPLC).

5.4 Determination of unbound aflatoxins in the buffer solution

Briefly, the pH of the clear supernatant was adjusted to 7.4 using 0.1M NaOH. Unbound aflatoxin in the supernatant was determined by the procedure suggested by Diaz and Smith (11) where the clear supernatant was analyzed for residual (unbound) aflatoxin without additional cleanup. The analysis employed fluorescence detector connected to HPLC (Shimadzu Corp) at a mobile phase flow rate of 0.8ml/min at a temperature of 28°C, through stationary phase column of size 5µm 4.6x150mm (Spherisorb ODS-1, Waters). Residual aflatoxins AFB1, AFB2, AFG1 and AFG2 were quantified at wavelengths of 363nm excitation filter and 440nm cut-off emission filter using the fluorescence detector (RF-10AXL SMN C20954406285).

5.5 Estimation of percent aflatoxin binding capacity

Aflatoxin binding capacity of a material was determined by the percent of AFB1, AFB2, AFG1 or AFG2 adsorbed into it. Thus the higher the aflatoxin binding capacity the lower the percent of unbound aflatoxin content in the buffer solution. The Percent binding capacity P_i of i^{th} TBM/Mycobind^R in binding j^{th} aflatoxin was determined using model equation (2).

$$P_i = (IAT_{ij} - UAT_{ij})/IAT_{ij} \times 100 \dots \dots \dots (2)$$

Where IAT_{ij} (ng/ml) is the initial concentration of j^{th} aflatoxin in the test-tube with i^{th} TBM/Mycobind^R; UAT_{ij} (ng/ml) is the residual (unbound) j^{th} aflatoxin in the test-tube with i^{th} TBM/Mycobind^R after the digestion period. The IAT_{ij} was considered to be the amount of aflatoxin recovered from positive control adjusted by subtracting the value obtained for the negative control. The UAT_{ij} was adjusted by subtracting residual aflatoxin amount obtained for the negative control of each individual TBM/Mycobind^R from the concentration of residual aflatoxin in the supernatant of the TBM/Mycobind^R spiked with aflatoxin solution.

5.6 Data analysis

5.6.1 Statistical analyses

Data analysis for percent mean binding capacity were analysed by GLM program of SAS (37) using the model equation (3).

$$Y_{ij} = X_i + X_j + e_{ij} \dots \dots \dots (3)$$

Where Y_{ij} = binding response (capacity) of i^{th} TBM/Mycobind^R in adsorbing j^{th} aflatoxin

X_i = binding effect due to the capacity of i^{th} TBM/Mycobind^R in adsorbing j^{th} aflatoxin

X_j = binding effect due to easy with which j^{th} aflatoxin is adsorbed to i^{th} TBM/Mycobind^R

e_{ij} = the error term due to i^{th} and j^{th} aflatoxin

The mean separation was done by Duncan procedure and the significance was declared at an alpha-level of 0.05.

Relationship between binding capacity of TBM and Mycobind^R in adsorbing aflatoxins and their chemical properties was determined by running correlation analysis between percent binding capacity of TBM and Mycobind^R and their relative chemical properties (elemental-oxide concentration and cation exchange capacity). Data were analysed by using MS-Excel.

5.6.2 Determination of aflatoxin binding capacity ratio of Mycobind^R to TBM

Binding capacity of Mycobind^R relative to a TBM as a ratio R was determined using the model equation (4)

$$R = \% \text{ binding capacity of RB} \div \% \text{ binding capacity of a TBM} \dots \dots \dots (4)$$

Conflict of interests

The authors declare that there is no conflict of interest related to this study.

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