TOXICITY OF AUTO-DETOXIFIED Jatropha curcas Linnaeus, 1753 KERNEL CAKE MIXTURES WITH BOVINE BLOOD (ADMJKC/BB) USING BRINE SHRIMP Artemia salina Linnaeus, 1758

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ABSTRACT: In present study, brine shrimp (Artemia salina L.) was used to determine the toxicity of auto-detoxified Jatropha kernel cake (JKC) mixed with bovine blood (ADMJKC/bb). The powdered-JKC was mixed with bovine blood (bb) at three ratios (1:1=X, 2:1=Y and 3:1=Z of JKC: bb) and the resultant mixtures processed using four protocols: Heated, Spread Dry = 1. Unheated Spread Dry = 2. Heated Spread Remoistened to 66% dry matter (DM) = 3 and Unheated Spread Remoistened to 66% dry matter DM = 4). The resultant 12 treatment combinations (X1, X2, X3, X4, Y1, Y2, Y3, Y4, Z1, Z2, Z3 and Z4) were placed in a Solar J. curcas auto-detoxification apparatus from where samples were retrieved periodically and evaluated for detoxification using the brine shrimp lethality test. There were no significant differences within the same mixture of the four protocols. However, there was a tendency for mean LC50 values to increase between the ratios. Specifically, Protocol 2 recorded a significant difference between between X2 and Z2 treatments, having 1:1 and 3:1 JKC: bb mixes respectively. Upon ranking the level of auto-detoxification, the most detoxified treatments (Z2 with LC50=4674 and Z4 with LC50 =3692) differed significantly from the least two (X1 with LC50=1383 and X2 with LC50=1459). Addition of bovine blood to JKC increased the dynamics of JKC auto-detoxification, probably due to the presence of some innate auto-detoxifying microbial inoculum and bovine blood which boost the rapid growth, development and succession of these microbes. Thus combining JKC with bovine blood is complementary for JKC auto-detoxification, with the most detoxified ingredients (Z2, Z4 and Y3) appearing most suitable for further development and testing as feed ingredient for farm animals.

Keywords: Auto-detoxification, Bovine blood, Feedstuff, Jatropha, Shrimp.

INTRODUCTION

Jatropha curcas has shown some promise as a crop from which biofuels can be produced without compromising human food needs (Nithiyanthanath et al., 2012; Maghuly and Laimer, 2013). However, despite global excitement on its potentials, at current levels of technology, value-addition from its by-products and co-products is necessary in order for viability of the sector to be ensured (Makkar et al., 2012). One of the by-products being evaluated in this regard is Jatropha kernel cake (JKC), the remnants obtained after extraction of oil from Jatropha kernels that have a potential for use as animal feed (Nithiyanthanath et al., 2012; Che Hamzah et al., 2020). Except for lysine, its nutrient profile surpasses the Food and Agriculture Organization of the United Nations (FAO)’s reference protein (Makkar et al., 1998).
However, it is toxic to humans and animals because of its phorbol esters and other anti-nutrients (trypsin inhibitors, lectins and phytate) and therefore requires detoxification prior to use (Sharath et al., 2014; Rodríguez-González et al., 2018).

Several methods targeting the larger industries and Jatropha farm holdings have been employed and categorized with varying degrees of success to detoxify JKC (Ewane et al., 2017). However, the need for simple cottage techniques for small and large-scale operations persists. Auto-detoxification of JKC has therefore been developed as a pro-poor and pro-rural detoxification method for JKC (Ewane et al., 2017).

Present study aimed to determine the toxicity of auto-detoxified mixtures JKC and bovine blood (ADMJKC/bb) on brine shrimp (Artemia salina L.). The strategy was to manipulate endogenous and environmental factors that can enhance self-detoxification in mixtures of JKC and bovine blood and identify the most promising treatment combinations. Bovine blood, often used to produce blood meal, highly supports microbial growth, and also represents one of the richest sources of lysine (NRC, 1994). Hence, the (ADMJKC/bb) mix shall enhance the nutrient profile and detoxification rate of JKC. Furthermore, the powdery ground JKC shall easily trap the liquid bovine blood and reduce the need for further processing of blood meal by small-scale farmers (Nithiyananthan and Francis, 2012).

MATERIALS AND METHODS

Study Location

The study used the facilities of the Teaching and Research Farm, University of Buea, Cameroon (located at 4.1667° N, 9.2333° E, 578 m asl). J. curcas seeds were harvested from farms, live fences and plantations in four of Cameroon’s five agro-ecological zones (Figure 1): Maroua in the Sudano-Sahel zone, Ngaoundere in the High Guinean Savana, Bamenda in the Western Highlands and Mamfe in the Humid Forest zone with a mono-modal rainfall pattern. Bovine blood was collected from the main abattoir in the town of Buea.

Preparation and pre-treatment of J. curcas kernel cake and bovine blood mix

J. curcas seeds were cracked open (using two hard boards) and the extracted kernels de-oiled using a hydraulic press. The J. curcas kernel cake (JKC) obtained was then finely ground using a plate mill and the powder homogenized by hand mixing before further mixing with bovine blood in three different proportions (1/1, 2/1 and 3/1).

Twelve combinations were developed for production and evaluation based on four processing protocols of JKC mixtures with bovine blood (bb) at three mix ratios (Table 1). The mixes were chosen by serially increasing the quantity of JKC in a fixed amount of bovine blood. This gave combination protocols from three ratio mixes and four auto-detoxification treatments. These protocols were inspired by previous studies on the effects of moisture (Abou-Arab and Abu-Salem, 2010), spreading (Schmidt and Hecker, 1975) and heat (Aregheore et al., 2003; Martinez-Herrera et al., 2006) on degradation of phorbol esters and/or associated anti-nutrients of JKC.

Each combination was replicated 4 times and placed in steel plates arranged on two tables within the performance SJADA (Ewane et al., 2017), operated at full detoxification mode with the air access inlet closed to the minimum level of 50 cm². Six of the 12 treatment combinations (Protocol code 3 and code 4: X3, Y3, Z3, X4, Y4 and Z4) were remoistened to 66% dry matter daily, while the other six (Protocol code 1 and code2: X1, Y1, Z1, X2, Y2 and Z2) were not remoistened. About 10 g of sample were collected weekly from each replicate to evaluate the level of auto-detoxification.

Table 1 - Treatment codes of JKC/bb mixing ratios, auto-detoxification and moistening cycles.

| Protocol Code | Auto-detoxification treatment of JKC: bb combination Protocol | JKC: bb mixing ratio |
|---------------|-------------------------------------------------------------|---------------------|
|               |                                                              | X (1:1)  | Y (2:1) | Z (3:1) |
| 1             | Heat, spread and dried - No remoistening                   | X1       | Y1      | Z1      |
| 3             | Heat, spread and dried - Remoistened to 66% dry matter     | X3       | Y3      | Z3      |
| 2             | No heat, spread and dried - No remoistening                | X2       | Y2      | Z2      |
| 4             | No heat, spread and dried - Remoistened to 66% dry matter  | X4       | Y4      | Z4      |

Figure 1 - Location map of Cameroon showing the agro-ecological zones where J. curcas seeds were collected.
Preparation of crude methanol auto-detoxified mixtures of JKC/bovine blood extracts (CMJKC/bbE)

A sample of about 40 g (10 g per replicate) was collected weekly from each treatment and analysed to determine the progress of auto-detoxification. The replicates of each treatment were pooled and further dried within the apparatus for another week, homogenised and powdered. From the powdered mass, 20 g were placed in an extraction bottle containing 200 mL methanol and the methanolic extract obtained by regularly stirring the whole for 72 h using a rotary stirrer. The extract was then filtered through Whatman No. 541 filter papers and the crude methanol auto-detoxified mixtures of JKC/bovine blood extracts (CMJKC/bbE) obtained by evaporation of the solvent using a rotary evaporator.

Brine shrimp bioassay

The brine shrimp lethality test (Meyer et al., 1982) was used, with some modifications, to test the toxicity of CMJKC/bbE. Brine shrimps (Artemia salina Leach) were hatched from eggs in rectangular dishes containing sea water. The dishes were sub-divided by a perforated barrier into two unequal chambers and kept under constant aeration. The bigger chamber receiving the eggs was in the dark while the smaller chamber receiving the hatched napuli in anticipation was under constant light. Forty-eight hours was allowed for the eggs to hatch and the phototropic napuli to mature. At this stage, the hatched napuli were drawn using a pipette and placed in a marked vial containing 4 ml of natural seawater. Prior to this, a CMJKC/bbE stock solution of 100,000 ppm was prepared by dissolving 200 mg of CMJKC/bbE in 2 ml of dimethyl sulfoxide (DMSO). The stock solution was then diluted serially with natural seawater to give a series of concentrations for testing (10,000, 1000, 100, 10 and 1 ppm). Three replicates of each concentration were prepared. From each predilution, 1 ml containing CMJKC/bbE, seawater and DMSO was added to the pre-marked vials containing 4 ml of natural sea water and 10 napuli to make a total volume of 5 ml. Negative controls were just dilutions of DMSO in seawater without CMJKC/bbE while un-detoxified whole Jatropha kernel cake served as the positive control.

The vials were incubated under light for 24 h, after which manual counting of dead and immobile napuli at bottom of vial commenced, against a lit background with the aid of a 3X magnifying hand lens. The mortality was calculated as the percentage ratio of the number of dead napuli to the total number of napuli tested after corrections to account for mortalities recorded in the control (Abbott, 1925) as shown in equation 1. Subsequently corrections were made for 0% and 100% as proposed by Ghosh (1984) and presented in equation 2 and equation 3, respectively.

\[
\text{Corrected mortality} \% = \frac{(M_{\text{obs}} - M_{\text{con}})/(100 - M_{\text{con}})}{x} \times 100 \quad \text{Eqn. 1}
\]

Where,

\[M_{\text{obs}} \text{ and } M_{\text{con}}\] are the respective observed and control mortalities.

\[
0\% \text{Corrected mortality} \% = 100x(0.25x n) \quad \text{Eqn. 2}
\]

\[100 \% \text{Corrected mortality} \% = 100x(n - 0.25/n) \quad \text{Eqn. 3}
\]

Where,

\[n\] is the number of test animals in each group.

Determination of lethal concentration

The lethal concentration of CMJKC/bbE resulting in 50 % mortality of brine shrimp (LC50) was determined from the 24 h counts by a plot of percentage of the shrimps killed against the logarithm of the CMJKC/bbE concentration and the best-fit line was obtained from the curve data by means of regression analysis (MS Excel version 7). The LC50 was derived from the slope of the best-fit line obtained.

Statistical analysis

Levene’s Test for Equality of Variances was performed on LC50. Also LC50 values were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means tested using Duncan’s Multiple Range Test (DMRT) (P<0.05). The software used was the IBM SPSS Statistics version 22 (IBM Corp. Released 2013). The most promising ADMJKC/bb treatments were selected after ranking the LC50 from the largest to the smallest values, with the largest values indicating the least toxicity. ANOVA and DMRT were used to detect significant difference of the LC50 values of ADMJKC/bb treatments.

RESULTS

The evolution of LC50 of crude methanol auto-detoxified mixtures of JKC/bovine blood extracts (CMJKC/bbE) with duration of incubation have been presented in Figure 2, while: comparison of 3 week means of LC50 of crude methanol auto-detoxified mixtures of JKC/bovine blood extracts (CMJKC/bbE) is in Table 2. By the third week of the trial, all the 12 treatments had LC50 values above 1000. This indicates they were all substantially detoxified. There were no significant differences (P>0.05) among the four Auto-detoxification treatment of JKC: bb combination protocols when compared within rations for each ratio of mixes.

However, there was a general tendency for mean LC50 values to increase across ratios as quantity of JKC increased in the mixture. The only exception was Protocol code3 (the Heat, spread and dried - Remoistened to 66% dry matter...
protocol). In Protocol code 3, the 2:1 ratio (Y3) is higher than both the 1:1 ratio (X3) and 1:3 ratios (Z3). In addition, Protocol code 2 (the no heat, spread and dried - no remoistening protocol) recorded a significant difference (P<0.05) between X2 and Z2 treatments which are compared across ratios.

The ranking of mean LC50 (Table 3) indicates that the most detoxified treatments were Z2=3,047.22, Z4=2,0130.67 and Y3=1,997.64, while the least detoxified were X2=653.65 X1=736.96 and Y1=1,092.93. The top two most detoxified treatments (Z2 and Z4) differed significantly (P<0.05) from the least two (X1 and X2).

Of all the treatments evaluated, the extent of lethality was found to be proportional to the concentration of the CMJKCE/bbE. High mortalities were recorded at 1000 ppm and 10,000 ppm, while lower mortalities were recorded at 1 ppm and 10 ppm. The graphical representations of the corrected brine shrimp mortalities in week 3 for the most and least detoxified treatments as well as the un treated whole Jatropha kernel cake control are shown in Figure 3.

![Graph showing the evolution of LC50 of crude methanolic auto-detoxified mixtures of JKC/bovine blood extracts (CMJKC/bbE) with duration of incubation.](image)

**Figure 2 - Evolution of LC50 of crude methanolic auto-detoxified mixtures of JKC/bovine blood extracts (CMJKC/bbE) with duration of incubation.**

**Table 2 - Comparison, LC50 of crude methanol auto-detoxified mixtures of JKC/bovine blood extracts (CMJKC/bbE) mean of 3 weeks**

| Protocol Code | Auto-detoxification treatment of JKC: bb combination Protocol | JKC: bb mixing ratio |
|---------------|-------------------------------------------------------------|---------------------|
| 1             | Heat, spread and dried - No remoistening                    | X1                  |
|               |                                                              | Y1                  |
|               |                                                              | Z1                  |
| 2             | No heat, spread and dried - No remoistening                 | X2                  |
|               |                                                              | Y2                  |
|               |                                                              | Z2                  |
| 3             | Heat, spread and dried - Remoistened to 66% dry matter      | X3                  |
|               |                                                              | Y3                  |
|               |                                                              | Z3                  |
| 4             | No heat, spread and dried - Remoistened to 66% dry matter   | X4                  |
|               |                                                              | Y4                  |
|               |                                                              | Z4                  |

**Table 3 - Ranking of LC50 of crude methanol auto-detoxified mixtures of JKC/bovine blood extracts (CMJKC/bbE)**

| Treatment | Mean ± sem | Group | RANK | Best fit equation | R²       |
|-----------|------------|-------|------|-------------------|----------|
| Z2        | 3047.22 ± 553.13 | b     | 1    | y = 22.568x - 32.795 | 0.8502   |
| Z4        | 2130.67 ± 563.01 | b     | 1    | y = 22.568x - 32.795 | 0.8502   |
| Y3        | 1997.64 ± 643.05 | a     | 2    | y = 23.337x - 32.795 | 0.8941   |
| X4        | 1796.54 ± 323.73 | ab    | 3    | y = 23.337x - 32.795 | 0.8941   |
| Y4        | 1794.53 ± 470.58 | ab    | 4    | y = 23.337x - 32.795 | 0.8941   |
| Y2        | 1700.64 ± 771.72 | ab    | 5    | y = 23.337x - 32.795 | 0.8941   |
| Z3        | 1498.18 ± 406.18 | ab    | 6    | y = 23.337x - 32.795 | 0.8941   |
| X3        | 1248.46 ± 815.19 | ab    | 7    | y = 23.337x - 32.795 | 0.8941   |
| Z1        | 1110.46 ± 652.12 | ab    | 8    | y = 23.337x - 32.795 | 0.8941   |
| Y1        | 1092.93 ± 783.83 | ab    | 9    | y = 23.337x - 32.795 | 0.8941   |
| X1        | 736.96 ± 844.73  | a     | 10   | y = 23.337x - 32.795 | 0.8941   |
| X2        | 653.65 ± 604.48  | a     | 11   | y = 23.337x - 32.795 | 0.8941   |
| JS Control| 736.96 ± 844.73  | a     | 12   | y = 23.337x - 32.795 | 0.8941   |

Values are means ± standard deviations of results obtained after 3 weeks. a,b,c: Mean LC50 values with different superscript differ significantly (P<0.05).
Figure 3 - Corrected brine shrimp mortality rates for different protocols and mixtures of Jatropha kernel subjected to different levels of auto-detoxification

DISCUSSION

According to Meyer et al. (1982), several extracts derived from natural products which had LC₅₀ ≤ 1000 μg/ml using Brine Shrimp Lethality Test (BSLT) were known to contain physiologically active principles while those with LC₅₀ values > 1000 ppm were considered inactive. This indicates that all the 12 ADMJKC/bb ingredients were substantially detoxified after 3 weeks of exposure in the SJADA. The evolution of the LC₅₀ values indicates that detoxification was more rapid with increasing content of JKC in the ADMJKC/bb ingredients. Two ingredients containing a ratio of 3:1 JKC: bb (Z2 and Z4) and two containing JKC: bb ratio of 2:1 (Y3 and Y4) all had LC₅₀ values above 1000 within the first week of auto detoxification compared to just one ingredient containing a ratio of 1:1 JKC: bb (X4). Nonetheless, this level of auto-detoxification for even the least ratio mix of JKC with bovine blood is remarkably rapid, when compared to LC₅₀ values of auto-detoxified JKC (ADJKC) ingredients produced solo without addition of bovine blood as reported in Ewane et al. (2017). In that study which was performed under similar conditions as the current study, it took 4 weeks for just 3 out of 14 ADJKC test ingredients on trial, to attain LC₅₀ values above 1000. Therefore, the addition of bovine blood to JKC probably changed the dynamics of auto-detoxification. According to Thomas (1988), in the live animal blood is generally a sterile medium, possessing innate bacteriostatic and bactericidal abilities. Such antibacterial effects are clearer with gram negative bacteria such as E-coil, and non-virulent strains of Vibro cholerae, Haemophilus influenzae, salmonellae and shigellae. Out of the animal, however, bovine blood is a rich nutrient medium encouraging the growth of several microbes. Adding bovine blood to JKC may therefore increase the range of microbes in the mixture which would rapidly increase the level of auto-detoxification. Kasuya et al. (2013) increased the level of bio-detoxification of JSC by adding 10% eucalyptus bark. From the data obtained, they deduced that the importance of adding eucalyptus bark served to balance carbon and nitrogen and decrease the fat content, thus resulting in improved fungal growth. They concluded that their results support the hypothesis that phorbol ester degradation occurs because of co-metabolism by the enzymes responsible for lignin de-polymerization.

Even though the differences were not significant (P>0.05), ADMJKC/bb produced by subsequent remoistening irrespective of whether they were preheated or not (Protocol code 3 and code 4: Z3, Y3, X3, Z4, Y4, X4) as a group were among the top most detoxified, while the ADMJKC/bb preheated without subsequent remoistening (Protocol code 1: X1, Y1 and Z1) were the least detoxified. Interestingly, the actual most detoxified, median most detoxified and actual least detoxified ADMJKC/bb ingredients were respectively Z2, Y2 and X2, from protocol code 2. These ingredients were unheated and un-remoistened; the difference between the three ingredients was the ratio of mixing JKC with bovine blood. They were respectively mixed at 3:1, 2:1 and 1:1 for Z2, Y2 and X2. The protocol code 1 (Z1, Y1, and X1) ADMJKC/bb ingredients which were the least detoxified as a group followed a similar detoxification ranking with Z1 as the most detoxified and X1 as the least detoxified for that group. What was common among the Protocol code 1 and code 2: Z1, Y1, X1 Z2, Y2, X2 groups was that they were all undergoing a form of unperturbed solid state auto-detoxification compared to their daily remoistened counterparts of Protocol code 3 and code 4 (Z3, Y3, X3 & Z4, Y4, X4). The only
difference is that while the Protocol Code 1 (Z1, Y1, X1) group was heated, the Protocol code 2 (Z2, Y2, X2) group was unheated. Similarly, while the Protocol code 3 (Z3, Y3, X3) group was preheated, the protocol code 4 (Z4, Y4, X4) group was unheated.

The differences observed among the various ratio mixes of JKC with blood therefore, probably indicate that the higher the ratio of JKC in the mixture, the higher the level of innate auto-detoxifying microbial inoculum. The hypothesis that needs to be verified is that “Microbes supporting auto-detoxification are present on the JKC and either act or wait for their opportunity to act in an ecological succession. Bovine blood provides a trigger (probably nutrients in simple molecules plus additional microbes) which boosts the rapid growth, development and succession of these microbes”. Evidence from an earlier study by Ewane et al. (2017) in support of this hypothesis is that powdered JKC treatments with neither addition of water nor yeast (un-moistened solar spread-US) and un-moistened diffuse daylight spread-UDC-) treatments were substantially detoxified, having LC50 of 63.89 and 5.22 respectively after four weeks of exposure in the Solar J. curcas Auto-detoxification Apparatus (SJADA) and Diffuse Daylight J. curcas Auto-detoxification Apparatus (DJADA) respectively. The observation that the ADMJKC/bb preheated without subsequent remoistening (Protocol code 1: X1, Y1 and Z1) as a group were the least detoxified is a pointer that the initial inoculum was probably destroyed by heat and a succession was slower to take off in the absence of added moisture. The abnormal higher ranking of Y3 over Z3 in Protocol code 3 is possibly an indication that release of nutrients and other factors from bovine blood as a proportion to available microbial inoculum to auto-detoxifying microbes in heat treated and subsequently remoistened ADMJKC/bb could also be a determinant to the rate of auto-detoxification.

Furthermore, the results of Chikpah and Demuyakor (2012), who observed that approximately 60% reduction in crude phorbol ester levels can be achieved within 21 days of spontaneous fermentation of J. curcas kernel meal, supports the hypothesis that JKC contains some innate auto-detoxifying microbial inoculum. As defined in Ewane et al. (2017), auto-detoxification is a self-detoxification process induced by endogenous and environmental factors including enzymes, microbes, sunlight, temperature, humidity and wind. It is the natural way to transform the toxic J. curcas seeds into an innocuous material. These processes take considerable time under natural conditions but their duration can be shortened by human manipulation. The results of this present study therefore highlight the possible role of microbes in the auto-detoxification process. Working with pure microbial cultures, some authors including Belewu and Akande (2010), Belewu et al. (2010), Bose and Keharia (2013), Kasuya et al. (2013) and Azhar et al. (2014) have confirmed the role of fungi in the detoxification of JKC, while others including El-Zelaky et al. (2011), Phengnuam and Suntornsuk (2013), Widiyastuti et al. (2013) and Chang et al. (2014) have confirmed the role of bacteria in the detoxification of JKC.

All the 12 ADMJKC/bb ingredients were substantially detoxified; however, the top three most detoxified ingredients (Z2, Z4 and X3) have been selected for further development. Any of the bottom nine (X4, Y4, Z3, X1, Y1, X1, and X2) may still be tested with animal models for further differentiation.

CONCLUSION

The addition of bovine blood to Jatropha Kernel Cake (JKC) changed the dynamics of auto-detoxification. Microbes supporting auto-detoxification are present on the JKC and either act or wait for their opportunity to act in an ecological succession. Bovine blood provides a trigger (probably nutrients in simple molecules plus additional microbes) which boosts the rapid growth development and succession of these microbes. Also, blood, which is the richest natural source of lysine, could supplement the reported lysine deficiency in JKC and the combined auto-detoxified mixtures Jatropha kernel and bovine blood; (ADMJKC/bb), would potentially become a better feed ingredient than either blood meal alone or JKC alone. Consequently, the top three most detoxified ingredients (Z2, Z4 and Y3) have been selected for further development and testing as feed ingredient for farm animals. However any of the bottom nine (X4, Y4, Z3, X1, Y1, X1, and X2) may still be tested with animal models for further differentiation.

DECLARARATIONS

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Authors’ contribution:
D.Ewane conceived the study, designed the study, collected data, contributed in data analysis, and writing the manuscript, coordinated the inputs of all the other authors; B.O.Oben and K.J.N.Ndamukong performed critical reviewing of the manuscript and supervision of the study; K.A.Etchu performed critical reviewing of the manuscript; E.E.Ehabe contributed in design of study, data analysis and writing the manuscript; J.M.Chah contributed in data collection and write up of the manuscript; K.F.Chah contributed in conception of the study, design of study, data analysis and critical reviewing of the manuscript; P.M.Oben performed critical reviewing of manuscript and coordinated the study.

Competing Interest
The authors have not declared any conflict of interest.

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