**In-vitro** lethality assay and performance of Sumain nutritional supplement on the liver enzymes and lipid profile of wistar albino rats

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Abstract

Sumain Nutritional Supplement (SNS) is a processed composite flour of groundnut, soya bean, guinea corn, and maize grains, and substituted at 0, 25, 50, 75, and 100% diet levels of a Commercial feed. In vitro toxicity study was conducted using Brine shrimp larvae, while in vivo performance of these diets was assessed using wistar albino rats in a 21-day feeding period. Mean body weight difference was measured. Liver extracts were assayed for alanine, and aspartate amino transaminase (ALT, AST) concentrations. Plasma extracts were assayed for haematological, and lipid profiles using standard methodologies. Results showed that SNS extract was safer than a reference tannic acid by 83%, with LC50 of 550. Compared with AST/ALT ratio of 1.17 for 100% Commercial feed, those of SNS-incorporated feeds ranged from 0.92 to 1.16, indicating no apparent damage to liver tissue. At all levels of SNS substitutions, WBC and PCV concentrations were within standard acceptable ranges, while results of MCV and Hb-related parameters exhibited very good biochemical indices of health status during the period of study. 100% and 75% SNS substituted diets particularly, showed potential to reverse thrombocytopenia. Increased Triglyceride (TG) level coupled with lowered levels of Cholesterol (LDL and TC), led to the recommendation of these two SNS diets as good nutraceuticals for human subjects having symptoms of malnutrition.

Keywords: Sumain Nutritional Supplement (SNS); Brine Shrimp Larvae Assay; Alanine amino transaminase (ALT); Aspartate transaminase (AST); Lipid Profile.

1. Introduction

Composite flour of grains prepared from different legumes, cereals and nuts is considered world-wide as good sources of proteins, carbohydrate, energy and dietary fibre. It also provides essential vitamins needed by the body. Furthermore, composite flour of these grains can be utilized in the production of good quality snacks, pan cakes and savoury foods [1]. The flour, through “diet-based strategies”, could also be employed in the management of protein-energy malnutrition disease and micronutrient deficiency. The strategy would be valuable to children and pregnant women especially in developing countries [2-3].

Leguminous crops, particularly soya bean (*Glycine max*) are valued locally and globally for their high protein and oil contents. Such crops are rich in essential amino acids, vitamins, calcium, and phosphorous, soyabean. They also contain isoflavone, which plays an important physiological role in the prevention of cancer and osteoporosis [4]. Similarly nuts such as, groundnut (*Arachis hypogaea*) possesses high dietary protein and oil contents enriched with minerals, vitamins
and essential amino acids [5]. However, cereals like maize (Zea mays) which serve as staple food in some parts of the world are peculiarly low in tryptophan and lysine [6] though; they contain high proportions of carbohydrate, essential fatty acids, vitamins E, K, C, B-complex, potassium as well as phenolic compounds, phytosterols and carotenoids [7]. Previous studies on nutritional properties of composite flour or multigrain flour in supplementary foods have revealed the possible utilization of multigrain and its nutritional benefits when incorporated in diets [1, 8-9].

In view of these benefits, diets were composed from a formulated Sumain product (a composite flour of cereals, legumes, and nuts) and substituted in a commercial feed at varied percentages. This study, therefore, investigated the nutritional effects on the enzymatic activities, haematological, and lipid profiles of experimental animals fed on these diets.

2. Materials and methods

2.1. Sample collection and processing

Commercially available dried yellow Soybean (Glycine max), red-skin variety of Groundnut (Arachis hypogaea), white Guinea Corn (Sorghum bicolor), yellow and white varieties of Maize (Zea mays) seeds were procured separately from Anyigba market, Kogi State, Nigeria. The experiments were conducted at the Biochemistry Laboratory of Kogi State University, Anyigba, Nigeria between March and October 2019.

Processing steps to effectively remove most anti-nutritional factors, as described by International Institute of Tropical Agriculture (IITA) [10] were adopted: The seeds were cleaned and sorted for wholesomeness. They were further subjected, where necessary, to soaking, draining, and air-drying e.g. Guinea Corn and Soya bean, before dehulling, toasting and grinding into powdery form called “Sumain Nutritional Supplement (SNS)”. Details of the treatments given are contained in Table 1.

2.2. Cytotoxicity bioassay using brine shrimp larvae

The assay was conducted using hatched brine shrimp (Artemia salina) larvae as described by Arogba and Matannisi [11]. Steps involved are described thus:

2.2.1. Preparation of sample extracts

With each diet sample type, 1 g was dissolved in distilled water (50 ml) to form the stock solution from which serial dilutions were made.

2.2.2. Hatching brine shrimp eggs

Equivalent sea water was prepared by dissolving 32 g of NaCl in 1000 ml of distilled water. Some 50 mg of brine shrimp eggs were added to 300ml of the artificial sea water and incubated for 24 hours under bright light while connected to an air-voltage pump that aerated the mixture.

After incubation, groups of 10 brine shrimp larvae were counted and transferred to different vials using a Pasteur pipette and volume was made up to 5 ml with the artificial sea water. Constant volume of 500 μl of each varied concentration (2000, 1000, 500, 250 and 125 μg/ml) of a diet sample type or reference potassium dichromate was added to the vials containing the shrimps. After 24 hours, the dead larvae were counted for the determination of the percentage lethality. The lethality endpoint of the bioassay was defined as the absence of controlled forward motion during 30 seconds of observation.

The percentage lethality of the shrimp for each concentration was calculated [11]:

\[
\%\text{Lethality} = \left( \frac{\text{Number of morbid shrimp larvae}}{\text{Number of surviving shrimp larvae in Control}} \right) \times 100.
\]

The LC\textsubscript{50} of the sample’s extract was calculated from linear regression curve of Percentage lethality against Log (concentration), when y = 50.
Table 1 Processing conditions for production of sumain nutritional supplement (SNS) flour mix

| Sample type     | Shelling / Sorting | Mild sprinkling of water | Soaking for 30 min / Draining | Air drying at ambient temp. | Dehulling by milling / Winnowing | Toasting | Manual dehulling | 60g eqv. Toasting time (min) | Milling into flour | Ratio in SNS mix | Weight (g) in Batch Production |
|-----------------|--------------------|--------------------------|-------------------------------|-----------------------------|---------------------------------|----------|------------------|-------------------------------|-------------------|-----------------|-----------------------------|
| Groundnut       | y                  | Y                        |                               | y                           | y                               | 1        | y                | 1                             | y                 | 1               | 300                         |
| Guinea corn     | Y                  | y                        | y                             | y                           | y                               | 2        | y                | 1.5                          | 2                 | 1.5             | 450                         |
| White maize     | Y                  | y                        |                               | Y                           | y                               | 0.5      | y                | 1                            | 300               | 300             |                             |
| Yellow maize    | Y                  | y                        |                               | Y                           | y                               | 0.5      | y                | 1                            | 300               | 300             |                             |
| Soya bean       | Y                  | y                        | y                             | Y                           | y                               | 1.5      | y                | 2                            | 600               | 600             |                             |
| **Total**       |                    |                          |                               |                             |                                 |          |                  |                               |                   |                 | **1.95 kg**              |

* y = Treatment given
2.3. Procurement and management of experimental animals

Male wistar rats weighing between 97 – 132 g were procured from the animal house of the Department of Veterinary Medicine, Benue State University Makurdi, Nigeria. They were acclimatized in clean rat cages at the Experimental Animal House of the Department of Biochemistry, Kogi State University, Anyigba for a period of seven (7) days at ambient temperature with 12-hour light and dark cycle. Within the period, they were fed with water *ad-libitum* and commercial "Broiler Top Feed Finisher" purchased from a supermarket at Anyigba.

2.4. Experimental design

2.4.1. Weight measurement

The weights of the rats were taken before and after twenty one (21) days feeding period. The percentage weight difference was calculated.

2.4.2. Diet substitution/Animal Grouping (Table 2)

Fifteen (15) rats was fed with commercial "Top Feed Finisher" diet and substituted with SNS at 0, 25, 50, 75 and 100% levels. Water was provided *ad-libitum*.

| SNS  | Comm. Feed (%) | Number of rats/ group |
|------|---------------|-----------------------|
| 0    | 100           | 3                     |
| 25   | 75            | 3                     |
| 50   | 50            | 3                     |
| 75   | 25            | 3                     |
| 100  | 0             | 3                     |
| Total| 15            |                       |

2.5. Blood sample collection

On the 22nd day, the animals were sacrificed by using chloroform in desiccator. The blood was obtained through cardiac puncture into heparinized (EDTA) bottles using 5ml syringe.

2.6. Assay of lipid profile

The lipid profile [Total Cholesterol, Triglycerides, and High Density Lipoprotein, and Low Density Lipoprotein] was determined by spectrophotometric technique, using enzymatic/colorimetric assay kits (Randox Laboratories, United Kingdom). However, Low Density Lipoprotein was calculated thus: LDL = TC – [HDL + TG/5].

2.7. Enzyme assay

The activities of Alanine transaminase (ALT) and Aspartate transaminase (AST) were analysed according to the method described by Reitman and Frankel [12].

2.8. Assay of haematological parameters

The packed cell volume (PCV), mean cellular volume (MCV), platelets (PLT), white blood cells (WBC), haemoglobin (Hb), and mean corpuscular haemoglobin concentration (MCHC) were all determined as described by Baker and Silverton [13], using automated haematology analyzer (Sysmex Kox1: Sysmex corporation, Kobe, Japan, Xp 300 Series, Code No AC580857)

2.9. Statistical analysis

Statistical analysis was conducted using SEM software available at miniwebtool.com. Results were expressed as mean ± standard error of mean (SEM), where expedient. Separation of mean was conducted for test of significance at (P = 0.05), where different alphabets denoted significant difference.
3. Results

Table 3 Lethality Rate of Brine Shrimp Larvae (expressed as LC50)

| Conc. (μg/ml) | Sample (%) | Tannic acid (%) | K2Cr2O7 (%) |
|---------------|------------|-----------------|-------------|
| 2000          | 73.3       | 93.3            | 100         |
| 1000          | 66.7       | 86.7            | 96.7        |
| 500           | 50.0       | 70.0            | 70.0        |
| 250           | 36.7       | 40.0            | 56.7        |
| 125           | 13.3       | 26.7            | 16.7        |
| LC50          | 549.5b     | 300.3a          | 274.4a      |
| SEM           | 87.70      |                 |             |

Values are expressed as mean of triplicate determinations. Values of LC50 with different superscripts on the same row are significantly different at p < 0.05.

The test sample of SNS was least toxic to Brine Shrimp larvae compared with the organic and inorganic reference samples employed. Their relative LC50 were significantly different (p<0.05).

Table 4 Mean weight of animals before and after feeding for 21 days (n = 3).

| Sample: Comm. Feed (%) | Before (g) | After (g) | Difference (g) | Difference (%) | SEM |
|------------------------|------------|-----------|----------------|---------------|-----|
| 0 : 100                | 97         | 146       | 49             | 51d           |     |
| 25 : 75                | 100        | 148       | 48             | 48cd          |     |
| 50 : 50                | 108        | 156       | 48             | 44c           | 4.7 |
| 75 : 25                | 121        | 160       | 39             | 32b           |     |
| 100 : 0                | 132        | 167       | 35             | 27a           |     |

Values with the same superscripts in the column are not significantly different at P > 0.05. SEM = Standard error of mean. Comm. Feed = commercial feed (Top feed Finisher).

Difference of 24% weight reduction was recorded between animals fed with 100% SNS sample and 100% commercial feed (p<0.05). However, there was no negative sign in loss.

Table 5 Enzyme concentrations at different diet substitutions

| Sample: Comm. Feed (%) | AST (IU/L) | ALT (IU/L) | AST/ALT |
|------------------------|------------|------------|---------|
| 0 : 100                | 53.1 ± 9.95b | 45.5 ± 12.50c | 1.17c   |
| 25 : 75                | 43.1 ± 6.10a | 47.0 ± 5.00c | 0.92a   |
| 50 : 50                | 42.5 ± 3.50a | 39.5 ± 2.50b | 1.08b   |
| 75 : 25                | 43.5 ± 4.50a | 37.5 ± 4.50a | 1.16c   |
| 100 : 0                | 42.5 ± 4.50a | 40.0 ± 6.00b | 1.06b   |
| SEM                    | 2.05       | 1.84       | 0.044   |

Values are expressed as mean ± SEM (n=3). Values with different superscripts on the same column are significantly different at p < 0.05.

AST concentration decreased significantly with SNS substitution in the commercial feed (p<0.05). and remained unchanged irrespective of level of substitution (p>0.05). Unlike AST, 25% SNS substitution did not influence ALT concentration, but higher SNS substitutions did. Consequently, AST/ALT varied and was SNS concentration-dependent. The least AST/ALT was observed at 25% SNS substitution in the commercial feed. (p<0.05).
Table 6 Haematological analysis

| Sample: Comm. Feed (%) | WBC (x10^3/L) | Hb (g/dL) | PCV (%) | MCV (dL) | MCH (pg) | MCHC (g/dL) | PLT (x10^3/L) |
|------------------------|---------------|-----------|---------|----------|----------|-------------|---------------|
| 0 : 100                | 6.5 ± 0.30<sup>b</sup> | 14.6 ± 1.60<sup>c</sup> | 47.0 ± 7.00<sup>b</sup> | 71.6 ± 0.25<sup>a</sup> | 18.0 ± 0.75<sup>b</sup> | 24.3 ± 0.30<sup>a</sup> | 225.0 ± 18.00<sup>c</sup> |
| 25 : 75                | 5.5 ± 2.00<sup>a</sup> | 11.8 ± 0.20<sup>a</sup> | 44.6 ± 0.60<sup>a</sup> | 71.5 ± 0.05<sup>a</sup> | 19.0 ± 0.55<sup>d</sup> | 26.5 ± 0.80<sup>c</sup> | 92.4 ± 6.40<sup>a</sup> |
| 50 : 50                | 6.9 ± 0.10<sup>c</sup> | 14.6 ± 0.05<sup>c</sup> | 55.9 ± 0.05<sup>d</sup> | 71.9 ± 0.10<sup>a</sup> | 18.4 ± 0.35<sup>c</sup> | 25.0 ± 1.00<sup>b</sup> | 89.0 ± 1.00<sup>a</sup> |
| 75 : 25                | 6.3 ± 0.50<sup>b</sup> | 12.6 ± 0.30<sup>b</sup> | 43.8 ± 3.80<sup>a</sup> | 73.3 ± 1.95<sup>b</sup> | 17.8 ± 0.60<sup>a</sup> | 24.4 ± 1.45<sup>a</sup> | 177.5 ± 35.50<sup>b</sup> |
| 100 : 0                | 7.1 ± 1.25<sup>c</sup> | 12.4 ± 0.90<sup>ab</sup> | 51.8 ± 1.50<sup>c</sup> | 73.7 ± 1.65<sup>b</sup> | 17.7 ± 1.15<sup>a</sup> | 24.0 ± 1.05<sup>a</sup> | 111.0 ± 5.00<sup>a</sup> |
| SEM                    | 0.28          | 0.59      | 2.29    | 0.46     | 0.24      | 0.45        | 26.77         |

Values are expressed as mean ± SEM (n=3). Values with different superscripts in the same column are significantly different at p<0.05.

Optimum levels of WBC, Hb, PVC, MCH, and PLT were recorded on substituting the commercial feed with 25% SNS (p<0.05). On the contrary, 100% commercial feed exhibited highest concentrations of WBC, PCV, and PLT (p<0.05) in the experimental animals.

Table 7 Lipid profile analysis

| Sample: Comm. Feed (%) | TC (mg/dL) | TG (mg/dL) | HDL (mg/dL) | LDL (mg/dL) |
|------------------------|------------|------------|-------------|-------------|
| 0 : 100                | 132.27 ± 2.52<sup>d</sup> | 224.77 ± 4.34<sup>b</sup> | 33.49 ± 1.31<sup>a</sup> | 53.83 ± 0.34<sup>d</sup> |
| 25 : 75                | 122.85 ± 4.40<sup>c</sup> | 219.34 ± 6.06b | 34.53 ± 1.30a | 44.45 ± 1.89c |
| 50 : 50                | 106.32 ± 2.52b | 102.92 ± 2.02a | 39.20 ± 0.78b | 46.53 ± 2.14c |
| 75 : 25                | 101.92 ± 3.98ab | 99.50 ± 3.57a | 41.47 ± 0.59c | 40.56 ± 3.86b |
| 100 : 0                | 96.48 ± 3.65a | 93.92 ± 3.58a | 43.10 ± 2.08c | 34.60 ± 4.92a |
| SEM                    | 6.72        | 30.24      | 1.89       | 3.19        |

Values are expressed as mean ± SEM (n=3). Values with different superscripts in the same column are significantly different at p < 0.05. SEM = standard error of mean.
There was no significant difference in the levels of TG and HDL of animals fed with 100% commercial feed, and those on 25% substitution (p<0.05). However, decreasing pattern of TC, TG, LDL, and in reverse, increasing HDL concentrations were observed as SNS substitution increased (p<0.05).

4. Discussion

Brine Shrimp larvae are considered as suitable samples for preliminary cytotoxicity assay in pharmacology [14-15]. Between 500 to 2000 μg/ml (Table 3) tannic acid and potassium dichromate were 20 to 30% more toxic respectively, to the brine shrimp larvae than with SNS extract. The reference samples had killed almost all the larvae at 2000 μg/ml. The result indicated that SNS sample was less toxic by 83% compared with tannic acid of similar organic origin used in this study. The observation was supported by the LC50 of 274 - 300 μg/ml for the reference samples, and 549 μg/ml SNS.

The percentage weight difference of the experimental animals in Table 4 showed that there was positive but decrease in weight gained as SNS substitution in diet increased from 25% to 100% (P < 0.05). However, none of the substitution levels had negative effect on weight gain which contrasts with observation from our previous similar design using 100% diet of defatted Mangifera indica, or Irvingia kernel powder [16].

Incidentally, the initial weights of the animals in this study had direct relationship with the increasing levels of substitution of SNS in the diets, which implied that age of animal and palatability were confounding factors on the percentage weight differences observed at the end of the 21-day feeding. Nevertheless, it was deduced that experimental animal with a mean weight of 115 g would have consumed SNS alone to an average weight gain of 53% relative to a diet of commercial feed alone. Furthermore, such animal would need about 40 to 43 days to attain maximum weight gain with 100% SNS diet.

The common enzymes used as indicators of hepatocellular damage or necrosis are the transaminases, particularly Aspartate Transaminase (AST) and Alanine Transaminase (ALT). Damage to the liver tissue leads to increase in serum or plasma levels of these enzymes, which also correlates with loss of functional integrity of the cellular membranes [17]. Both enzymes are quantified by similar colorimetric technique but ALT is more liver specific, and the AST/ALT ratio of greater than 1.5 is indicative of severe damage [18].

Table 5 showed that AST concentration significantly decreased with SNS substitutions, even at 25% level compared with the Control of 100% commercial feed (p<0.05). The decrease, however, was insignificant between the substitution levels, indicating that SNS had no adverse effect on muscle or liver tissues of the experimental animals. On the contrary, ALT levels of Control and the 25% substitution were not significantly different (p=0.5) but were significantly higher than those of 50, 75 and 100% SNS substitutions. Significant fluctuations were observed with the ALT concentration among these substitutions (p<0.05), and such ALT variations even in 24-h life-cycle of animals were reported as normal [18].

The results further showed significant variations in the ALT/AST ratios of animals fed with substituted SNS feeds (p<0.05) that ranged lower than 1.17 observed with the animals fed on 100% commercial feed, indicating that SNS substitution in the diets had no adverse effect on the liver tissue. Hence, its consumption could help in maintaining the integrity of liver cells and protect against non-alcoholic steatohepatitis (NASH) or hepatic necrosis such as cirrhosis, toxic or viral hepatitis, carcinoma and obstructive jaundice [19].

Table 6 showed that different levels of SNS substitution had different significant effects on the parameters analysed (P<0.5). However, some levels showed similarity with specific parameters of the Control, that is, the Commercial feed; examples include 75% substitution for WBC, 50% for Hb, and 25% for MCV. Furthermore, the Hb, MCH, MCHC, and MCV of both the 75 and 100% SNS diets were similar (P>0.5). These observations implied that SNS diets especially at high concentrations could maintain cellular integrity and osmolarity, as well as the capability to boost WBC production in case of tissue damage resulting from immune infections. The inverse mathematical relationship in the formula, MCHC= Haemoglobin concentration X 100/PCV [20] was in agreement with the results obtained. Furthermore, normal ranges of WBC and PCV as defined by Sood [21] were also obtained in this study, to affirm the positive effect of SNS-based diets in nutrition.
On the contrary, the PLT concentration increased proportionally with SNS substitution, but were significantly lower than that of the Commercial feed alone (P<0.5). Basal PLT concentration of 150,000 had been described as normal [21]. The results obtained, therefore, indicated that the animals purchased for the study had symptom of thrombocytopenia, which was reversed by only the 75% SNS-substituted diet and 100% Commercial feed in the period of study.

From Table 7, it was observed that TC and LDL decreased with increasing SNS sample substitution, and inversely correlated with HDL. TG concentration using the 100% Commercial feed decreased significantly (P < 0.05) on 25% SNS substitution but subsequent decrease from 50 to 100% SNS substitutions showed no significant difference (P>0.05). In similar vein, the observed increase in HDL concentration with SNS substitution was not significantly different between 75 and 100% substitutions.

Furthermore, less than 150 mg/dl of TG was classified as 'desirable', and 150 – 500 mg/dl as 'borderline high' [22]. It implied that diets of 50 to 100% SNS substitution gave desirable TG content of 94 – 103 mg/dl. The concomitant decrease in total cholesterol and LDL concentrations with the SNS substitutions compared with the 100% Commercial feed was a reflection of the more superior lipid composition of SNS product, in respect of its phytosterol and fatty acid profiles. Findings from our independent study indicated that SNS had a high ratio 7.6 : 1.0 of unsaturated : saturated fatty acids, PUFA/SFA of 3.22 against expected >0.4, and ω6/ω3 of 0.71 against expected <4.0 in conformance with FAO/WHO [23]. This study, therefore, has shown that 100% SNS or 75% SNS when complemented with micronutrients, was nutritionally acceptable; a product recommendable to human subjects with symptoms of malnutrition.

5. Conclusion
SNS sample extract had LC₅₀ of 550 and was comparatively less toxic than tannic acid by 83%. In vitro and in vivo analyses of SNS-based diets, relative to a 100% commercial feed, had no adverse effect on tissue integrity of particularly the liver. It was nutritionally acceptable to the experimental animals employed, and potentially capable of reversing thrombocytopenia. Hence, 100% SNS or 75% substitution in diets complemented with micronutrients is recommended as nutraceutical for human subjects with symptoms of malnutrition.

Compliance with ethical standards

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Disclosure of conflict of interest
The authors declare no conflict of interest.

Author’s contributions
Akpala envisioned the study and participated in the conduct of the experiments with the co-authors (Amlabu, Amodu, and Arogba). In addition, Arogba designed the methodologies, supervised the experimentation, analysed the data, and scripted the article.

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