

UNDER PEER REVIEW

1 **Research paper**

2 **Influence of Water – washed *Neem* fruits (*Azardirachta indica A.***

3 ***juss*) on**

4 **Haematology and Serum biochemical indices of**

5 **West African Dwarf sheep**

6

7 **ABSTRACT**

8 A 4-week feeding trial was conducted to investigate the effect of supplementation of water-washed
9 neem fruit (*Azardirachta indica A.juss*) in diets on haematological and serum biochemical indices of
10 WAD sheep. Three diets were formulated to contain water-washed *neem* fruit at graded levels of
11 inclusion; 0% (control), 5% and 10%. Twelve WAD sheep between 12 – 15 months old were
used.

12 The experimental animals were divided into 3 groups of 4 sheep per group and randomly
assigned to

13 the 3 dietary treatments. Variations in values obtained for haemoglobin, Red Blood Cell (RBC),
Mean

14 Cell Volume (MCV), Mean Cell haemoglobin (MCH), White Blood Cell (WBC) and Lymphocyte of
15 WAD sheep fed diets 1, 2 and 3 respectively. Haematological parameters measured were
significantly

16 ($p < 0.05$) different while other parameters were similar among the treatment. The serum urea,
serum

17 creatinine, serum glucose and serum alkaline phosphatase for WAD sheep fed diets 1, 2 and 3
18 respectively were significantly ($p < 0.05$) different. Other serum biochemical indices measured were
19 similar among the treatments. The results obtained from this study showed that the inclusion of
water

20 washed *neem* fruit at 5 % and 10% is not recommended. This is as a result of the depression in the
21 haematological parameters measured. Considering the serum biochemical parameters measured,
all

22 the values reported fell within the normal physiological range except for the serum enzyme. The
23 fluctuation observed in the haematological parameters and serum enzyme could probably be
24 physiological responses to the antinutrient factors present in the *neem* fruit.

25

26 Key words: Water-washed; *neem* fruit; haematology; serum biochemistry; sheep

27

28 **ABBREVIATIONS**

29 WAD: West African Dwarf; RBC: Red Blood Cell; MCV: Mean Cell Volume; MCH: Mean Cell
30 Haemoglobin; WBC: White Blood Cell ; Hb: Haemoglobin values; PCV: Packed Cell Volume;
AST:

31 Aspartate Transaminase; ALT: Alanine Transaminase; ALP: Alkaline Phosphatase

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Comment [MN1]: Check you mean west African dwarf goat(WADG) anything like WAD sheep in literature?

Comment [MN2]: ????

Comment [MN3]: recast sentence

Comment [MN4]: significantly capital ($P < 0.05$)

Comment [MN5]: recast, while there were no significant ($P < 0.05$) difference observed in other parameters.

Comment [MN6]: This statement contradicts your initial report of no significant difference in other parameters . verify

Comment [MN7]: Recast, there were no significant ($P < 0.05$) difference between the groups and the control on other parameters

Comment [MN8]: These sentences should be used as discussion, therefore move to discussion section.

Comment [MN9]: Remove this section, rather first write the words in full before their abbreviation in bracket in the Text.

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38 1. INTRODUCTION

39 Nutrition has been an indispensable aspect of livestock production. Meeting the nutritional needs
40 as

41 well as the requirements of livestock, conventional and established feedstuffs have been implicitly
42 utilized in feeding livestock. Although plethora of research findings on the excellent performance
43 of

44 these conventional feedstuffs fed to livestock have been extensively documented but competition
45 between man and livestock for cereals and cereal product has made these ingredients
46 uneconomical

47 for continuous use as livestock feed. In order to reduce cost of feed which constitutes a significant
48 fraction of total cost of production, focus has been on the search for cheaper and readily available
49 feedstuffs with little or no competition with man's dietary demands [1] and [2].

50 Until recently, search for alternatives led to a compendium of non-conventional feed resources
51 available for feeding livestock. Among the promising non-conventional feedstuffs is *neem*
52 (*Azadirachta indica*).

53 *Neem* is a tropical evergreen tree, native to several countries; its seed/fruit contains a high protein
54 content ranging between 300 to 400g/kg, and anti-nutrients (i.e. Triterpenoids, Azadirone,
55 Nimbin,

56 and Salanin) responsible for its pungent smell as well as its bitter taste. Interestingly, the products
57 from the neem plant such as; leaves, fruits, seeds, kernels, oil and cake have demonstrated some
58 health benefits in human subjects [3]. On the other hand, prolonged consumption of neem over a
59 55 period of time could lead to kidney damage [4]. In spite of its anti-nutrients, possibility of neem as
60 56 animal feed source and its toxicological effects have been widely reported for monogastrics [3];
61 [5].

62 The possibility of neem seed/fruit as an animal feed source to elucidate normal responses from
63 monogastrics can be attributed to reduction in level of anti-nutrients by processing such as
64 washing.

65 Emphasis has been on performance responses of monogastrics (specifically rabbits and broiler
66 60 chickens) to diets supplemented with *neem* seed cake/fruit but with little or no documentation on
67 the

68 implications of feeding water-washed *neem* fruit on blood indices of WAD sheep. Hence, the
69 62 objective of this study is to evaluate the haematological and serum biochemical responses of
70 WAD

71 63 sheep to water-washed *neem* fruit based diets.

64 1. MATERIALS AND METHODS

65 Preparation of samples

66 *Neem* fruits were harvested from matured tree during the late rainy season period (September –
67 66 October). The fruits were sundried and the sun-dried fruits were then soaked in water twice within
68 24 hours for about 4 times within 2 days, then, decantation at the end of every 24 hours. Then the
69 fruits

70 were sundried and milling was done after the 48th hour.

71 2. 1 Experimental procedure

72 A total of twelve West African Dwarf (WAD) sheep were purchased and allowed two weeks
73 adaptation to the experimental pens. Routine management practices were carried out. Sequel to
74 the

75 73 start of experiment, sheep were offered weighed amounts of experimental diets for two weeks to
76 74 ensure acclimatization of the ewe to the experimental diets. At the beginning of the experiment,
77 sheep

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Comment [MN16]: No clinical investigation of the experimental sheep before study?

75 were randomly allotted to three treatments with four ewes per treatment housed individually. Weighed
 76 amount of the experimental diets offered to the sheep comprised of guinea grass (*Panicum maximum*),
 77 the basal forage at 70% of the ration, while the supplementary feed (cassava peels, soybean meal, corn
 78 bran, water-washed neem fruits, and molasses) supplied the balance of 30% Table 1. The addition of
 79 water-washed neem fruit was done at graded levels of 0%, 5% and 10% for treatments 1, 2 and 3
 80 respectively in replacement of corn bran in the diet. Water was made available throughout the
 81 duration (28days) of the feeding trial.
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Table 1: Gross composition of experimental diets (g/100g)

DIETS

86 Ingredient (%) Treatment 1 Treatment 2 Treatment3

87 Cassava peels 60.00 60.00 60.00

88 Soybean meal 20.00 20.00 20.00

89 Corn bran 10.00 5.00 0.00

90 Neem fruit 0.00 5.00 10.00

91 Molasses 10.00 10.00 10.00

92 Total 100.00 100.00 100.00

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2.2 Blood sample collection

96 Blood samples were collected from the jugular veins of the sheep at the 28th day, into a sterilized glass

97 tube containing EDTA (Ethylene Diamine Tetra Acetic Acid) and another glass tube without anti-coagulant for haematological and serum biochemical assays respectively. Blood samples for serum

99 assay were centrifuged and serum was decanted and freeze stored at -10°C until analysis.

2.3 Haematology

101 The packed cell volume (PCV) and haemoglobin (Hb) were determined using micro haematocrit method and cyanmethaemoglobin method respectively [6]. Erythrocyte count (RBC) and leukocyte

103 count (WBC) were determined using the improved Neubauer haemocytometer after the appropriate

104 dilution [7]. Other blood corpuscular constants (MCV, MHC and MCHC) were calculated.

2.4 Serum biochemistry

106 Serum glucose was determined by the O-Toluidine method using acetic acids [8]. Serum urea

107 was determined by urease method and creatinine by Folin-wu filtrate methods as described [9]. Serum

108 total protein was determined by Biuret method as described [10] and [11], while albumin was

109 determined using the BCG (Bromocresol green) method [12]. The Globulin was obtained by

110 subtracting Albumin from Total Protein. Serum Cholesterol, Triglycerides were measured using

111 appropriate laboratory kits [13; 14]. Aspartate transaminase (AST), Alanine transaminase (ALT)

and

Comment [MN18]: There is a confusion here, how many variants did you investigate. If it was neem as stated then there shouldn't be variation in the corn bran ration given to the groups. The only variation should be the graded neem ration given to the groups. Except you are investigating both corn bran and neem. VERIFY.

Comment [MN19]: Small letters: ethylene diamine tetra acetic acid

Comment [MN20]: Centrifuged without mentioning allowing the blood to clott for how long?

Comment [MN21]: As described by who?

Comment [MN22]: As described by who?

112 Alkaline phosphate (ALP) activities were determined using spectrophotometric methods [15 and 16].

113 **2.5 Statistical Analysis**

114 Data obtained were subjected to analysis of variance [17] and where significant difference occurred

115 means were separated using Duncan Multiple range test of the same package.

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Table 2: Chemical Composition of the Experimental Diets

122 Nutrient (%) Treatment Treatment Treatment

123 1 2 3 SEM

124 Crude protein 10.65 10.76 10.97 7.00

125 Crude fibre 19.90 19.79 19.69 37.00

126 Ether extract 4.15 4.20 4.18 0.90

127 Ash 7.50 7.69 7.59 10.00

128 Nitrogen free extract 57.80 57.56 57.57 45.10

129 Neutral detergent fibre 53.83 54.05 54.17 77.00

130 Acid detergent fibre 33.56 33.98 33.93 60.00

131 Acid detergent lignin 4.26 4.77 4.68 40.00

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151 **3. RESULTS AND DISCUSSION**

152 **3.1 Haematological indices of WAD ewes fed water – washed neem fruit**

153 Presented in Table 3 is the haematological response of ewes fed water – washed neem fruit

154 supplemented diets.

Comment [MN23]: were

Comment [MN24]: why are there variations in the chemical composition of the treatments given to different groups. The compositions should be same such that any difference could be attributed to the only existing variant which is the neem composition. As it is you cannot make definite conclusion because you already have different compositions which could have attributed to your result findings.

Comment [MN25]: Be consistent with choice of words WAD sheep or ewe

Table 3: Haematological response of West African Dwarf sheep fed water-washed neem fruit

supplemented diets.

Parameters Treatment Treatment Treatment SEM

1 2 3

PCV (%) 29.67 26.50 23.00 0.81

Hb (g/dl) 10.00_a 8.83_{ab} 7.67_b 0.27

RBC (x10⁶/mm³) 4.81_{ab} 5.84_a 4.08_b 0.22

MCV (fl) 70.75_a 49.95_c 58.39_b 1.05

MCHC (%) 33.33 33.33 33.33 0.003

MCH (pg) 23.58_a 16.65_c 19.46_b 0.32

WBC (x10⁹/mm³) 6.81_a 6.59_{ab} 6.38_b 0.28

NEUT (%) 49.00 43.25 45.25 1.04

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LYMPH (%) 48.50_b 55.25_a 53.00_{ab} 0.93

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MONO (%) 1.50 0.50 1.00 0.24

EOSI (%) 1.00 1.00 0.75 0.27

abc – Means along the same row with different superscripts are significantly ($p < 0.05$) different.

SEM-Standard error of mean, PCV- packed cell volume, Hb-haemoglobin, RBC-Red blood cell,

MCV-Mean cell volume, MCHC- Mean cell haemoglobin concentration, MCH- Mean cell

haemoglobin, WBC- White blood cell, NEUT-Neutrophils, LYMPH- Lymphocytes, MONO-

Monocytes,

EOSI-Eosinophils

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The values obtained in this study 184 for PCV were 29.67%, 26.50% and 23.0% for T₁, T₂ and T₃

185 respectively. Hb values ranged from 10.00g/dl to 7.67g/dl for T₁ to T₃. Significant differences

($P = 0.05$) were obtained with increasing levels of water – washed neem fruit in the diet. Also, the

187 values obtained for RBC were 4.81. The haematological values obtained in this study including

188 control or T₂ and T₃ alone (Table 3) were outside the standard range recommended for clinically 189 healthy sheep (ewes) [6]. The mean cell haemoglobin concentration (MCHC) of animals on treatment

190 2 and 3 were not significantly ($P = 0.05$) different from the control group. The 33.33% obtained across

191 the treatments for MCHC was within the 30.0 - 34.4% reported [6] for clinically healthy ewes. The

192 MCHC values have been reported to be the most accurate and absolute values that indicate anaemic

193 condition in animal [18 and 19].

194 The MCV and MCH values observed in this study were above the normal range (28.4 –

Comment [MN27]: Delete repetition

Comment [MN28]: Suggest you reverse your tabular presentation, place the parameters on horizontal axis with their units in brackets and the treatment groups on the vertical axis. The standard error of mean was it for which group or was it meant for the 3 groups?. You may need to Re- analyze for each group on different parameters

Comment [MN29]: Do not place a range, state the result of each group eg 10.0g/dl T₁; 8.83g/dl T₂ and 7.67g/dl T₃.

Comment [MN30]: $P > 0.05$

Comment [MN31]: Only? What of results of T₁ and T₂.

Comment [MN32]: ?????

Comment [MN33]: This contradict s your initial statement made on line no 187-189 considering the importance of MCHC in diagnosis of anaemia.

195 31.6fL) and (9.20 – 9.80 pg) respectively reported for healthy ewe in temperate climate [6]. The
196 higher mean cell volume (MCV) and the higher mean haemoglobin (MCH) values recorded in the
197 present study when compared to [6] in the temperate climate could be attributed to climate and
198 breed

198 differences.

199 The non significant value of packed cell volume (PCV) of the ewe on water-washed *neem*
200 fruit supplemented diets relative to the control group was an indication that the animals were not
201 anaemic. The PCV values of the ewe on the test diets were not significantly different from the
control

202 group and this confirms the report [20] and [19] that nutrition affects blood profile of animals. The
203 haemoglobin (Hb) and Red blood cell shows significant differences in the values obtained from
the

204 ewe across the treatments and this was not in agreement with the findings [20 and 19] that
nutrition

205 affects the blood profiles of animals. The values of the white blood cell (WBC) and their
differential

206 counts were within the normal range reported for healthy ewe [6] except for neutrophils. White
blood

207 cell in animal posses' phagocytic function [21 and 19] and differential WBC counts were used as
an

208 indication of stress response and sensitive biomarker crucial to immune function [22]. The
results of

209 the WBC in the present study clearly shows that the ewe on 10% water-washed *neem* fruit
210 supplemented diets were stressed hence the significant reduction in leukocyte counts.

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**Table 4: Serum biochemistry of West African Dwarf 224 sheep fed water-washed neem fruit
225 supplemented diets.**

226 Parameters Treatment Treatment Treatment SEM

227 1 2 3

228 Total protein (g/dl) 5.34 5.88 6.09 0.21

229 Albumin (g/dl) 2.72 3.84 2.96 0.21

230 Urea (mg/dl) 15.31_b 15.42_b 19.27_a 0.25

231 Creatinine (mg/dl) 0.73_b 1.04_a 1.08_a 0.02

232 AST (μ.i/l) 7.42 7.54 6.95 0.31

233 ALP (μ.i/l) 30.31_b 61.34_a 61.96_a 1.57

234 ALT (μ.i/l) 14.57 13.98 14.42 0.19

Comment [MN34]: Be consistent ewe or WAD
sheep

235 Cholesterol (mg/dl) 59.51 64.16 64.81 1.21
 236 Triglyceride (mg/dl) 48.87 42.80 47.34 1.83
 237 Glucose (mg/dl) 70.66^a 58.61^b 57.51^b 1.35
 238 *abc – Means along the same row with different superscripts are significantly (p<0.05) different.*
 239 *SEM-Standard error of mean, AST-Aspartate amino transferase, ALT-Alanine amino transferase,*
 240 *ALP-alkaline phosphatase.*
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255 3.2 Serum biochemical indices

256 Results of serum biochemical indices of the **ewe** fed water-washed *neem* fruit supplemented
 257 diets in this study are presented in Table 4. The serum urea, creatinine and serum glucose of animals
 258 were significantly ($P=0.05$) influenced by the dietary treatment. In the study, increases were observed
 259 in serum urea and creatinine levels and corresponding decrease in the serum glucose level. **This**
 260 **result** agrees with [23] that animal will normally fall back on the stored energy in the muscles when
 261 there is reduction in blood glucose level. The urea and creatinine concentration in the blood were used
 262 as kidney function test [24 and 19]. The non **significant** value of serum total protein and albumin of
 263 the **ewe** on water-washed *neem* fruit supplemented diets can be compared with the earlier report of
 264 protein retained in animals [25 and 19] and all the values obtained for the 2 parameters were
 265 within the normal range reported [6] for a normal healthy **ewe**. This result contradicts the depression in
 266 plasma protein concentration that was reported [26] using Murrah Milch buffaloes on the
 267 replacement of concentrate mixture with 15 and 20 parts of *neem* seed cake and [27] in growing goats on
 268 replacement of concentrate mixture with 15 – 25 parts of water-washed neem seed kernel cake.
 269 The serum cholesterol and triglyceride values obtained in this study were within the normal range
 reported

Comment [MN35]: Be consistent

Comment [MN36]: $P>0.05$

Comment [MN37]: This argument does not support your result, or are you trying to explain the cause of emaciation in the animals which you did not mention in the first instance.

Comment [MN38]: What are you saying about the kidney function test? What is the interpretation of your result?

Comment [MN39]: ($P<0.05$)

Comment [MN40]: What is your explanation for no change in total protein of treated animals.

270 for healthy ewe [6]. The values for the 2 parameters were found to be increasing with the increasing
271 dietary levels of water-washed *neem* fruits fed to the WAD sheep (ewe). This observation probably
272 may suggest an increase in the lipid mobilization. The serum Aspartate amino transferase (AST) and
273 serum Alanine amino transferase (ALT) values observed in this study were above the normal range
274 (40.0 - 123 μ .i/l) and (25.0 - 70 μ .i/l) respectively reported for healthy ewe [6]. The non significant
275 decrease in serum AST and increase in serum ALT, activities of the animal on treatments 2 and 3
276 could indicate an improvement in liver function due to hepatoprotective activities of *neem* [28
277 19]. The serum alkaline phosphatase (ALP) values were significant and were not within the normal
278 range (μ .i/l) reported [6] for clinically healthy ewe. |

279 4. CONCLUSION

280 The results obtained from this study have shown that the inclusion of water-washed *neem*
281 fruit at 5 % and 10% is not too deleterious to the animal but any further increase may result into
282 anaemic condition. However, at 5% and 10% level of inclusion is safe and economical in diets
283 sheep production with no adverse effect. Further studies at lower levels of inclusion may be
284 evaluated.
285 The fluctuation observed in the haematological profile and serum enzyme could be normal.

286

287 COMPETING INTRESTS

288 Authors have declared no conflicting interests exists
289

290 AUTHORS' CONTRIBUTION

291 This work was carried by the three authors. Author TOO wrote the protocol, coordinated
292 the execution of the study. Author MKA designed the study and the statistical analysis
293 while author TOF worked on the field and carried out the laboratory analysis. All the
294 authors read and approved the final manuscript.

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Arrange your references according to the journal's instruction for authors.

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Comment [MN41]: If neem have hepatoprotective activity, then what is responsible for increase in serum liver enzymes which are only present in cases of inflammation, injury or degenerative changes in the liver.

Comment [MN42]: Please your conclusion contradicts your result findings, first neem have deleterious effect on the ewes, it caused decreases in PCV, Hb and RBC of the treated groups. The level of decrease corresponds to the quantity of neem administered. Secondly, there were increases in both the creatinine and urea levels even the liver enzymes, signifying harmful effects on the two vital organs of the body, kidney and liver.

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