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Trypanostatic Effect of Millet-garlic Blended Diet on Rats Infected with *T. b. brucei*

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Authors' contributions

This work was carried out in collaboration among all authors. Author MHG designed the study and wrote the protocol. Authors AYK, AA, SA and LMH managed the animals, collected all data, performed the statistical analysis and wrote the first draft of the manuscript. Authors YG, FMM and AAQ did the literature search and also wrote part of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The nutraceutic effect of millet-garlic blend against trypanosomiasis in *Trypanosoma brucei brucei* challenged rats was investigated in this study.
Experimental Design: The experimental rats were grouped into A, B, C, D, E, F, G, H, I, and J in a complete randomised clinical trial design. Each group consists of three (3) rats randomly assigned.

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Place and Duration of the Study: The research work was conducted in the Department of Animal Production Technology, Federal College of Wildlife Management, New Bussa, Niger State, Nigeria. The four weeks feeding trial aspect of the work was carried out in the Biochemistry and Nutrition laboratory while the parasitological aspect was conducted in the veterinary clinic of the department.
Methodology: Albino Wister rats were randomly grouped into ten (10) treatments consisting of three (3) rats in each treatment (i.e A-J). Graded level of inclusion of blended millet- garlic at 5, 10, 15, 20, and 25% level was fed to treatments A-E. Group F, served as the control group, hence, were fed the conventional feed only. To group G, pulverised millet only (without garlic added to it) was fed to it, while group H, were fed with conventional feed supplemented with garlic only. Another group that was fed with the supplemented diet at the average inclusion rate of 15% for 3 days prior to infection forms group I. In the 10^{th} group (i.e. J), the feeding formular was similar to group I but the feeding period prior to infection was extended to 7 days. Thereafter, parasitaemia was monitored at two days interval. In addition, the effects of non prophylactic feeding (at 25% inclusion) and seven days prophylactic feeding (at 12.5% inclusion) of the experimental diet on the haematological parameters of experimental rats was also investigated in separate groups.
Results: While all the groups non-prophylactically fed the experimental diet at various inclusion level died before the 21^{st} day into experiment, the group fed the supplemented diet prophylactically for three (3) days died on the 27^{th} day into the experiment. Interestingly, the group fed prophylactically for seven days at 12.5% level of inclusion level exhibited trypanostatic activity, A significant difference (P = 0.05) in the haematological parameters between the group prophylactically fed the millet-garlic blend and the group on non-prophylactic treatment was also observed.
Conclusion: This study demonstrate the efficacy of prophylactic feeding of millet-garlic blend as a trypanostatic agent against proliferation of the parasites in the experimental animals and as a haematopoietic agent.

Keywords: Trypanostatic; trypanosome; prophylactic; functional foods; haematopoietic.

1. INTRODUCTION

Human African trypanosomiasis (HAT), literally known as sleeping sickness, is a disease that renders the patients unproductive and liability to rest family members and in most cases end up killing the patients. In situations where medical intervention in the form of prognosis, diagnosis and treatment is completely lacking very high mortality rate is certain [1] African Trypanosomiasis is a disease that affects both humans and animals and which can be passed from an infected individual to a healthy one through the bite of tse tse fly of *Glossina spp.* The mode of transmission and factors that determines the extent of the disease burden are environmentally dependent. Parasites of the genus *Trypanosoma* are known to be the major causative agents of this dilapidating disease. These parasite, are mostly found living and replicating outside the cells of their victims (i.e extracellularly), in the extracelluar, interstitial fluids and the blood [2]. Concerted but unrewarding effort by the livestock farmers in the affected areas has been reported to annually cost them about 35million US dollars in the course of the purchase of approximately 35 million doses of trypanocidal drugs by the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC), [3]. When summed up together, the amount spent (or invested?) by the NGO'S, donor agencies and the governments in the affected countries annually in the treatment of the disease and control of the vector is in the region of 1.2 billion US dollar [3]. This figure translate to economic losses as provision of basic amenities and infrastructural developments would have otherwise been achieved in such poverty stricken countries using such huge amounts [4] .

Trypanosomiasis like its associated neglected tropical diseases are strongly associated with poverty, most pronounced in environments devastated by civil conflicts or unrests and all confined to a common geographical areas, where they tend to coexist. This (trypanosomiasis) is a disease that has been devastating humans along with their livestocks for centuries. As a result of significant improvement in the health care delivery system in so many countries ,coupled with the improve living conditions and hygiene, the scourge of this disease is not felt as previously. That this disease and its allies (NTD'S), are no longer a source of concern to the developed nations, they continue to wreck havoc in the war tone and poverty stricken parts of the developing and

(especially) the underdeveloped countries, where about one million people in rural areas or slums in the cities periphery remain victims. This diseases devastate this group of people and remain unheard due to the fact that such group of affected individuals have little political voice. Increasing drug resistance, inability to develop vaccines (due to antigenic variations), serious unwanted sides effect and the high cost of the drugs are problems that warrants for search of a more novel drug entities [5]. The fact always remains that, diseases that are more prevalent in the poverty stricken areas of the world will certainly not attract any appreciable investment in research and development of new and effective drug entities by the pharmaceutical companies, as the target individuals cannot afford to pay for such profit dependent products [1].

Nutraceutical is a functional food that aids in the prevention and/or treatment of diseases and or disorders. The vulnerability of humans and other animals to infectious diseases is most of the time tied to the deficiencies of molecules that are very essential for the uptake and utilisation of yet other molecules (metabolomics), which are so essential but not synthesised by the human's or animal organs. Nutraceuticals are known to naturally contain phytochemical constituents that can have long-term health promoting or medicinal qualities which cannot be provided (synthesised) by the body itself. Phytochemicals are naturally occurring biochemicals that give plants their color, flavor, smell, and texture, which may help prevent diseases. While some of the Nutraceuticals serve essentially as therapeutics on patients of specific diseases, bulk of them are useful as health giving food supplements for general population [6].

Allium sativum, commonly known as garlic, is a species in the onion genus, *Allium.* Its close relatives include the onion, shallot, leek, chive,[7] and rakkyo [8]. This plant (particularly the bulb) has been shown to be a native of central Asia with a record of being used by humans for over 7,000 years [9]. The phyto-components of garlic has also been reported to be effective against such diseases and disorders as respiratory infections, cancer, diabetes, arteriosclerosis, cholesteraemia, liver disorders, parasites and worms infection of the gastro intestinal tracts and many more [10].

Pearl millet (*Pennisetum glaucum*) also known as spiked millet, is also called Gero (in Hausa), Yanji (in Kambari),bajra (in India) and bulrush millet [11]. Pearl millet consist of many cultivated races as opposed to the insinuation that it is a single species. It is a native of drier part of tropical western Africa and was, some 2000years ago introduced into the eastern and central Africa and to India, where because of its excellent tolerance to drought, became established in the drier environments. Depending on the annual rainfall recorded in a particular area, the height of the pearl millet plant may range from 0.5 to 4 m and the grain colour can be nearly white, pale yellow, brown, grey, slate blue or purple. The grains are usually ovoid in shape and could measure up to about 3 to 4 mm long, which by comparism, is larger than the size of other species. The size of the pearl millet kernel is about one-third that of sorghum. The relative proportion of germ to endosperm is higher than in sorghum. Sorghum and millet grits are steamed to produce a coarse and uniformly gelatinized product called couscous in many West African countries,. This (couscous) can be consumed fresh or can be dried; in its dried form, it can be stored for a long period of time ranging to six months and above. The dried product is reconstituted in hot water and milk or sauce is added to make a good delicacy [12].

Aside of the couscous, porridges are other major foods made from millet in several African countries. Porridges could either be thick or thin in consistency depending on the mode of production in a particular area. These porridges carry different local names. Thick porridges are called uguli (Kenya, United Republic of Tanzania, Uganda), to (Burkina Faso, the Niger), tuwo (Nigeria), aceda (the Sudan), bogobe, jwa ting (Botswana) and sadza (Zimbabwe). Thin porridges are called uji (Kenya, United Republic of Tanzania), ogi or koko (Nigeria, Ghana), edi (Uganda), rouye (the Niger, Senegal), nasha (the Sudan), rabri (India), bota or mahewu (Zimbabwe) and motogo (Botswana). In order to improve the nutritional value of the traditional porridges, millet flour, millet malt, pigeon pea and groundnut are mixed in different proportions. In Uganda, strongly sweet alcoholic drink called bushera is prepared by boiling and fermentation of un-germinated millet flour (Blackherbals.com) [13].

This work set to investigate the effect of Concurrent and prophylactic feeding potentials of various inclusion levels of millet-garlic blend in experimental animals challenged with *Trypanosoma brucei brucei.*

2. MATERIALS AND METHODS

2.1 Samples Collection and Preparation

Ten kilogram (10 Kg) of *Pearl millet* along with One kilogram (1 Kg) *Allium sativum* (Garlic variety) were purchased from the Sabo/Wawa Market in Borgu Local Government Area of Niger State, Nigeria.The millet was soaked for 2-3 days until it begins to sprout. It was then drained and allowed to dry at room temperature until the moisture content is about 10-15%. One thousand gram (1 Kg) of Garlic was crushed using pestle and mortar and then mixed with the dried Sorghum in the ratio 10:1. The mixed sample was then pulverised and sieved through. Conventional rat feed was obtained from the sales point. Graded level inclusion of the supplementary diet at 5, 10, 15, 20, and 25% were made.

2.2 Experimental Design

A total of 30 Albino Wister rats weighing between 125 – 143 g were purchased from Food and Nutrition Dept. of the Niger State Polytechnic, Zungeru, Niger State, Nigeria. They were acclimatized for seven (7) days in the Biochemistry and Nutrition Laboratory of Animal Production Technology Department, Federal College of Wildlife Management, New Bussa, Niger State, Nigeria. and used in the experiment. The animals were grouped into ten (10) treatments each containing 3 rats (i.e. A- J). Complete randomized clinical trial design was used in the experiment

2.3 Feeding of Experimental Diet

One hundred and sixty eight gram (162 g ± 2.32) of conventional feed was found to be the mean quantity enough to feed a group of three rats for one day and hence used to feed the control group (i.e Group F), while water was given to all the groups *ad libitum.* From this 168 g, 5% of it was replaced with the millet-garlic mixture and was fed to Group A (8.4 g), Group B 10% (16.8 g), Group C 15% (25.2 g), Group D 20% (33.6 g) and Group E 25% (42 g). Group G, were fed 12.5% of the conventional feed supplemented with the pulverized sorghum (21 g) only. Group H, were fed with conventional feed supplemented with 12.5% of garlic (21 g) only. Group I, were fed 15% (25.2 g) inclusion for three (3) days prior to infection and lastly Group J were fed 15% as in Group I but the feeding period prior to infection was extended to seven (7) days.

2.4 Trypanosomes Used and Infection of the Animals

The species of the parasite used was *T. b. brucei.* The strain of the parasites was obtained from the Nigerian Institute for Trypanosomiasis Research (NITR) Kaduna, Kaduna State. The parasite was maintained in our Laboratory by serial passage to healthy rats. Blood was collected by cardiac puncture with ethylenediaminetetraacetic acid (EDTA) coated syringe from a heavily infected mouse and immediately diluted with physiological saline to serve as the inoculums. Healthy mice were infected intraperitoneally (*i.p.*) with 0.02 ml of the diluted blood containing 1×10^6 trypanosomes. Monitoring of parasitaemia was done every 48 h by microscopic examination of blood sample taken from the tail of infected mouse pre-sterilised with methylated spirit. In each group, minimum of five fields was viewed using electric Microscope and the average was determined and employed for further analysis using Herbert's and Lumsden Table [14].

2.5 Determination of Hematological Parameters

The hematological components including haemoglobin, haematocrite, red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), granulocyte count (GRA), lymphocytes, platelate count, mean platelate volume (MPV), plateletecrit and platelete distribution weight (PDW) were determined in the group prophylactically fed for 7 days prior to infection in which clearance was achieved, using the automated haematologic analyzer as reported by Bashir et al. [15].

2.6 Statistical Analysis of Experimental Data

Data obtained in this study was subjected to a one-way analysis of variance (ANOVA) to derive mean values of parasitaemia which was compared with least significant difference.

3. RESULTS AND DISCUSSION

Findings that emanated from this piece of research work clearly indicates that, nutraceutical potentials greatly abound in most of the local foods commonly found in the developing and the third world countries, particularly the Sub

Saharan Africa for which drug supply against some diseases devastating the areas are either grossly inadequate or the price is exorbitantly high (beyond the reach of the poor majority). This potentials could not be unconnected to the presence of pharmacologically active compounds that abound in these cheap, readily available and affordable food substances.

The result from this study clearly indicates that feeding rats with diets mixed with Millet-garlic (as functional foods) non prophylactically at the inclusion level of 5-25% does not exert any trypanocidal nor trypanostatic effect in all the groups fed the functional food (Fig. 1). It is also apparent that, feeding garlic or millet solely at an inclusion level of 12.5% does not exert any trypanocidal/ trypanostatic effect. It is also worthy of note that, while the animals non prophylactically fed various inclusion levels of millet-garlic blend survived longer for Twenty(20) days at 25% inclusion level, the group prophylactically fed for three(3) days at 12.5% inclusion level survived for up to twenty eight (28) days (Fig. 2). However, an interesting observation made is the survival of the experimental animals prophylactically fed millet-garlic blend for seven days for up to forty days and beyond while the level of parasitaemia remained sustainably low (Fig. 3). The observation recorded from Fig. 3 prompted further investigation on the haematological parameters and the result indicated a possible immuno-modulatory role played by one or more of pharmacologically active compounds in either the millet, garlic or both (Table 1).

Millet has been found to be among the plants that are very rich in a compound called nitriloside, These compounds are involved in an amazing biochemical process that brings about the suppression of cancer cells. Therefore, whenever people that subsist on foods/ diets made from millets eat their traditional natural diet, their intake of nitrilosides is high, and their cancer incidence tends to be low [16].

This compound (nitriloside) found in millet is a crystalline structure which contains two units of glucose (sugar), one of benzaldehyde, and one of cyanate, which are tightly bonded together. This nature of bonding in the compound, brings about chemical inertness which in effect does not bring any significant effect on human tissues [17]. This nitriloside molecule is broken down by an enzyme, β-glucosidase into cyanate and benzaldehyde and for this reason is sometimes referred to as the “unlocking enzyme”. This molecule (nitriloside) exerts its anti cancer activities, when the enzyme (β-glucosidase) in the presence of water, breaks it down into cyanide and benzaldehyde, which are released and which are known to be highly toxic by themselves. The most interesting fact about this biochemical process is that the β-glucosidase is not found anywhere in the body except at the

Table 1. Effects of on prophylactic feeding and 7-days prophylactic feeding of the experimental diet on haematological parameters of rats

Parameters	Control rats	12.5% 7 days prior to infection	Non prophylactic 25% inclusion
WBC (x 10^{9} /L)	2.91 ± 1.11^{a}	5.38 ± 1.18^{b}	4.29 ± 0.90^{c}
Granulocytes (%)	0.76 ± 0.70^{a}	0.70 ± 0.24^{a}	2.19 ± 0.80^{b}
Lymphocytes (x 10^{9} /L)	2.08 ± 0.60^{a}	3.80 ± 0.58^{b}	2.86 ± 0.79^{b}
MCTC (x$10^{9/L}$)	0.18 ± 0.08^{a}	0.12 ± 0.005^{a}	0.26 ± 0.37^{b}
RBC(x10^{12}/L)	2.79 ± 0.10^{a}	3.19 ± 0.05^{a}	1.48 ± 0.77^{b}
Hematocrite (L/L)	0.34 ± 0.01^{a}	0.32 ± 0.00^{a}	0.21 ± 0.003^{a}
Hemoglobin (g/L)	93.23 ± 1.20^{a}	96.78 ± 1.85^{a}	95.00 ± 5.03^{a}
MCH (pg)	28.96 ± 1.59^{a}	30.50 ± 0.35^{a}	28.67 ± 1.31^{a}
MCHC (g/L)	385.33 ± 2.74^{a}	430.87 ± 3.60^{b}	389.00 ± 2.98^{a}
MCV (fL)	97.23 ± 1.20^{b}	92.93 ± 1.85^{b}	71.33 ± 0.33^{a}
RCDW-cv (fL)	39.13 ± 0.08^{a}	36.23 ± 3.16^{a}	41.43 ± 0.93^{a}
RCDW-sd (fL)	18.00 ± 0.36^{a}	14.97 ± 0.11^{a}	165.80 ± 0.36^{a}
PC (× 10^{9}/L)	334.68 ± 36.08^{a}	803.55 ± 6.83^{b}	769.00 ± 26.50^{c}
MPV (fL)	8.70 ± 0.09^{a}	8.89 ± 0.35^{a}	14.00 ± 0.30^{b}
Plateletecrit (L/L)	0.63 ± 0.15^{a}	1.46 ± 0.15^{b}	1.68 ± 0.17^{b}
PDW (%)	19.63 ± 0.41^{a}	17.99 ± 0.03^{a}	25.83 ± 1.11^{b}

LY: Lymphocytes; MCTC: Mid cell total count; RCDW-cv: Red cell width coefficient of variation; RCDW-sd: Red cell width standard deviation; PC: Platelate count; Values are mean ± SEM of 5 determinations. The values along the same row with different superscripts are significantly different (P < 0.05)

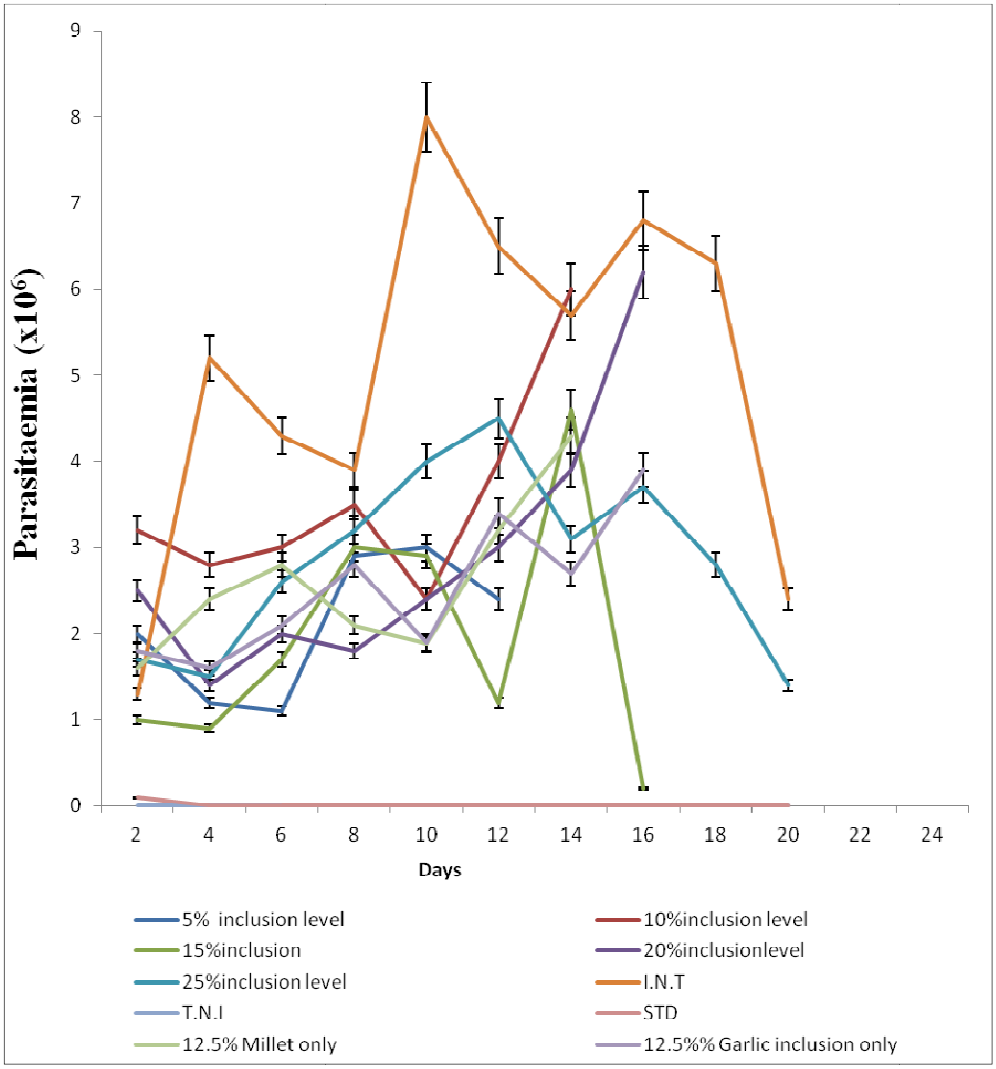

Fig. 1. Trypanocidal effect of various inclusion levels of millet-garlic blend in the conventional feed

cancer cells, where it is always present in large quantities, as much as one hundred times that of the normal cells. The result is that the nitriloside molecule is unlocked at the cancer cell site, releases its poisons to the cancer cell, and only to the cancer cells. In two separate studies reported by Ernst and Krebs; Tariq and Sawandi, [16;17], nitriloside therapy in patients with breast cancer, prostate cancer, lung cancer, skin cancer, and colon cancer, has proven to be very effective with a relatively low toxic side-effects. Also, in a study reported by Helen and Mutuku [18] *Pennisetum glaucum* (pearl millet) was found to contain phytochemicals such as Tannins, Flavonoids, Terpenoids, Glycosides, Phenols and Steroids and was at the same time found to be active against *Serrattia marcescens, Samonella typhi, Proteus vulgaries and Staphylococcus epidermis.* The presence of wide range of phytochemicals in pearl millet may be an explanation as to why the grain posses varied array of therapeutic effect [18]. Records have also indicated that, due to the presence nirilosides, millet and its related crops have been employed in the treatment of disorders such as: sickle cell anaemia, rheumatism, arthritis, and high blood pressure [16].

Records has it that for thousands of years, garlic has been used as a remedy for many different ailments and disorders such as intestinal disorders, flatulence, worms, respiratory

infections, skin diseases, wounds, and symptoms of ageing. It is a blood thinner that helps to lower both high blood pressure and blood triglycerides which confer on it, its anti-arthritic property. This property has been attributed to the activities of diallyl sulphide and thiacremonone present in garlic [19]. Garlic also has anti-inflammatory properties [20]. Several population studies also showed an association between an increased intake of garlic and a reduced risk of certain cancers, including colon, stomach, and oesophagus, pancreas, and breast cancer and this anti cancer activities has been found to be connected to a compound allyl sulphides it contains [21,22]. One reason for the increased incidence of breast cancer among women who eat large quantities of meat is due to presence of a compound called PhIP (2-amino-1-methyl-6-phenylimidazopyridine which forms components of Heterocyclic amines (HCAs).This PhIP is rapidly transformed into DNA- damaging compounds and the Diallyl sulfide (DAS), one of the many sulfur-containing compounds in garlic, has been shown to inhibit the transformation of PhIP into carcinogens [23,24]. The inhibition of the production of Phase I liver enzymes CYP1A1, CYP1A2 and CYP1B1 (by feed-back mechanism), that transform PhIP into activated DNA-damaging compounds by DAS, blocks this transformation by decreasing the production of these liver enzymes. Additionally, garlic is a triple threat against infections, offering antibacterial, antiviral, and antifungal properties. Garlic has even been found to be effective at killing antibiotic-resistant bacteria, including MRSA.

The fact remains that If not for Nok et al., and Nose et al., [25,26] that reported on the in-vitro trypanocidal effect of garlic, there is no report on the either trypanostatic nor trypanocidal effect of the two functional foods in -vivo and the extent of our search has not come across any report on trypanostatic/cidal effect of millet or the blend of the two crops.

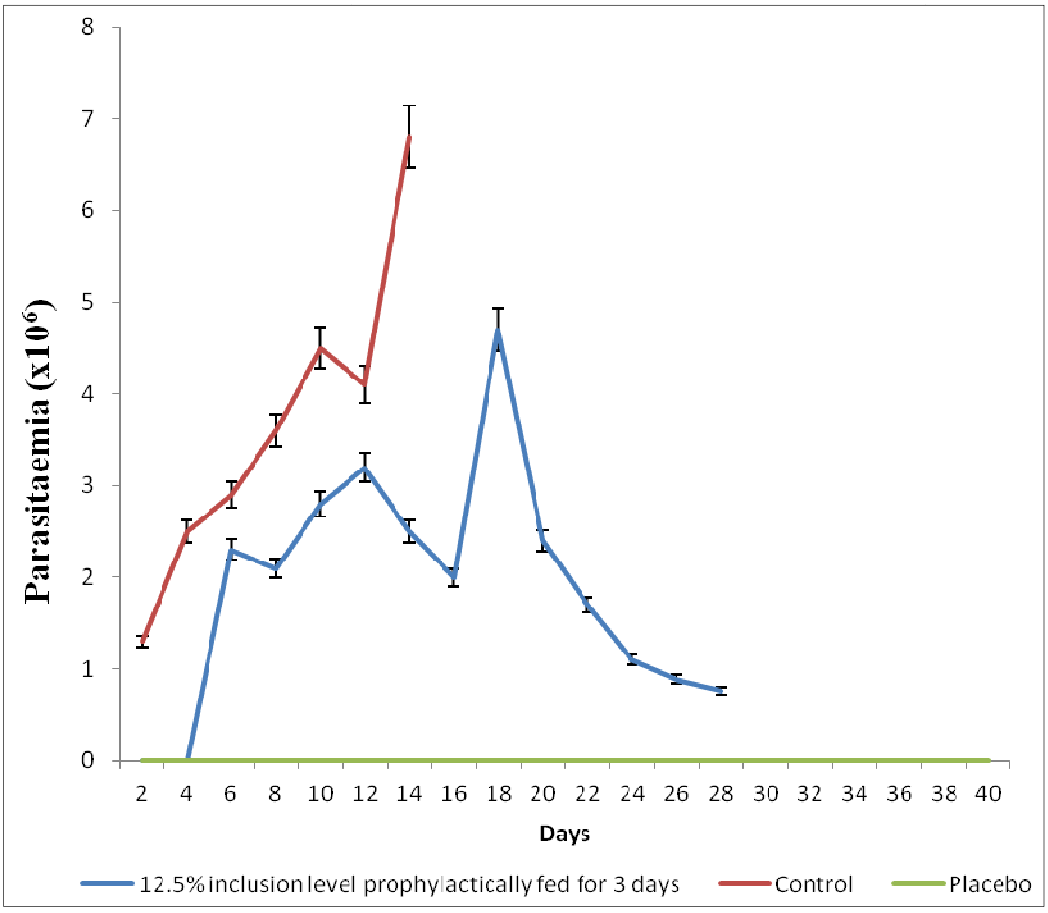

Fig. 2. Effect of three days prophylactic feeding of millet-garlic blend at 12.5% inclusion level

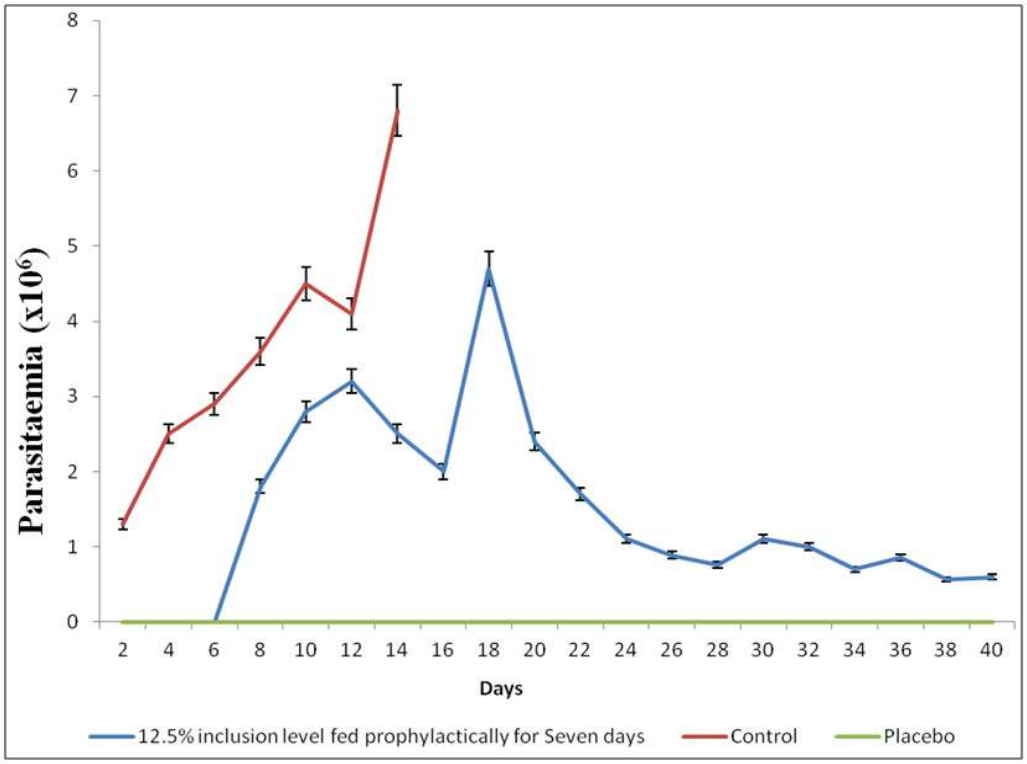

Fig. 3. Trypanostatic effect of seven days prophylactic feeding of millet-garlic blend at 12.5% inclusion level

It is difficult to speculate the mechanism by which these functional foods exhibit their trypanostatic activity since the active ingredient(s) were not isolated. However, accumulated evidence [27,28] suggest that many natural products exhibit their antitrypanosomal activity by virtue of their interference with the redox balance of the parasites acting either on the respiratory chain or on the cellular defenses against oxidative stress. It is also known that some agents act by binding with the kinetoplast DNA of the parasite. However, observation has been made that virtually most of the drugs with trypanocidal activities were observed to also display some cytotoxic (Anti cancer) and antitumor activities [29,30,31]. Based on this fact it could speculated that the nitrilosides present in millet and which is known to be hydrolysed at the sites of high β-glycosidase production (i.e the cancer cells and concentration of bacterial infestation) to yield sugar, free hydrogencyanide, benzaldehyde or acetone. These are highly toxic by themselves but become at least a hundred times more poisonous than either of them separately when they act synergistically. So, trypanosomes like the cancer cells are known to be efficient Glucose oxidisers and hence might posses similar hydrolysing enzyme that may eventually break and subsequently lead to the release of the afore mentioned oxidants that will exert oxidative stress that will weaken and eventually suppress the continuous growth and proliferation of the parasites as observed in our findings. Pearl millet are also reported to contain condensed tannins [18,32,33], which might be an additional reason for its antimicrobial activity.

On the possible reason why trypanocidal activity was not achieved could be due to the fact that, the only millet flavonoids reported are flavones [34]. While 3-deoxyanthocyanidins and proanthocyanidin contained in sorghum [35] is lacking. The mechanism of proanthocyanidins toxicity against microbes is related to inhibition of hydrolytic enzymes, interactions to inactivate microbial adhesions and cell envelope transport proteins, and non-specific interaction with carbohydrates [36]. This may be the reason trypanocidal effect was not achieved as in the case of Garba et al. [29] similar work on sorghum. Also, glucosylvitexin, glucosylorientin, and vitexin detected by Reichert [37] in pearl millet and which are responsible for the yellow–green discoloration of millet flour at basic pH

might be possible antagonists to the hydrolytic products of nitrilosides and hence reduced oxidative stress induction.

The interplay of several factors acting either individually or synergistically contributes to the development of haemolytic anaemia in human and animal trypanosomiasis. Most common among these factors are erythrocyte injury caused by lashing action of trypanosome flagella, undulating pyrexia, platelet aggregation, toxins and metabolites from trypanosomes, lipid peroxidation and malnutrition [38]. The possible reason for the significant difference (*P=0.05)* in virtually all the haematological parameters between the prophylactic treatment and the control in Table 1 may not be unconnected to the fact that the measurement was carried out at Phase III (Recovery phase) when the trypanosome number has drastically reduced hence lowering the incidence of mechanical injury to erythrocytes, adhesion of erythrocytes, platelets and reticulocytes to trypanosome surfaces via sialic acid receptors, all which may lead to damages to erythrocyte cell [38].

4. CONCLUSION

The result from this study clearly indicates that feeding rats with diets mixed with Millet-garlic (as functional foods) non prophylactically at the inclusion level of 5-25% does not exert any trypanocidal nor trypanostatic effect in all the groups fed with functional food (Fig. 1). It is also apparent that, feeding garlic or millet solely at an inclusion level of 12.5% does not exert any trypanocidal/ trypanostatic effect. It is also worthy of note that, while the animals non prophylactically fed various inclusion levels of millet-garlic blend survived longest for Twenty (20) days at 25% inclusion level, the group prophylactically fed for three (3) days at 12.5% inclusion level survived for up to twenty eight (28) days while the animals prophylactically fed for seven days survived up to 120days and beyond.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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