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Sorghum –Garlic Blended Diet Prophylactically Fed to Experimental Rats Prevent the Establishment of *trypanosomes* in the *T. b brucei*- Infected Animals

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Authors' contributions

This work was carried out in collaboration between all authors. Author MHG designed the study, wrote the protocol and supervised the work. Authors LMH, BJL and SA carried out all laboratories work and performed the statistical analysis. Author MI managed the analyses of the study. Author YG wrote the first draft of the manuscript. Authors A. Abubakar and AA managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This research work set to investigate the effect of feeding a mixture of two functional foods with a view to ascertain their therapeutic effect against *Trypanosoma brucei brucei* in challenged rats.

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Experimental Design: Complete randomized clinical trial design was used in the experiment. The rats were blocked for sex and grouped into A, B, C, D, E, F, G, H, I, and J respectively. Each group consists of three (3) rats.

Place and Duration of the Study: The research work was conducted in the Biochemistry and Nutrition laboratory in the Department of Animal Production Technology, Federal College of Wildlife Management, New Bussa, Niger State, Nigeria. Four weeks feeding trial was employed in the cause of the experiment**.**

Methodology: A total of 30 albino Wister rats were randomly grouped into ten (10) treatments each containing 3 rats (i.e. A- J). To groups A- E, feed containing graded level of inclusion of blended Sorghum- Garlic (10**:**1) at 5, 10, 15, 20, and 25% level was fed to them. Group F, were fed the conventional feed only (i.e. negative control). Group G, were fed solely with the pulverised *sorghum bicolor* (without Garlic added to it). Group H, were fed with conventional feed supplemented with garlic only. Another group of three rats, (i.e. group I) were fed with the supplemented diet with the average inclusion rate of 15% for 3 days prior to infection. To the final group (i.e. group J), as in group I but the feeding period prior to infection was extended to 7 days. Parasitaemia was thereafter monitored on two days interval while the effects of non prophylactic feeding (25%) and seven days prophylactic feeding (at 12.5%) of the experimental diet on the haematological parameters of rats was also determined.

Results: Seven days prophylactic feeding of the experimental animals with 12.5% inclusion of the sorghum-garlic prior to their infection with the parasites proves to be effective against the establishment and subsequent proliferation of the parasites. Also, significant difference (P = 0.05) in the haematological parameters was observed between the group prophylactically fed the sorghum-garlic supplemented diet for seven days period at 12.5% inclusion and the group fed at 25% inclusion and infected just a day after.

Conclusion: This study demonstrate the potency of prophylactic feeding of sorghum-garlic (functional foods) as nutraceutical against trypanosomiasis and as a haematopoietic agent.

Keywords: Sorghum; garlic; trypanosome; nutraceutical; Haematopoietic agent.

1. INTRODUCTION

According to the World Health Organization (WHO) human African trypanosomiasis (HAT) is one of the deadliest disease found on the earth [1,2]. No vaccine is developed, and there is no commercial interest in developing new drugs [3]. Three of the four main drugs (i.e Suramin (1916); Pentamidine (1937); Melarsoprol (1946)) used in treatment were registered before 1950. The most recent drug (i.e Eflornithine (1977)) was initially developed as an anti-cancer drug, only by coincidence found to be effective against HAT. HAT is a disease which the affects poorest of the poor [4]. Despite the fact that man is the natural mammalian host for trypanosomes, studies on animals revealed the involvement of numerous wild animals such as non-human primates, reptiles, antelopes, and wild bovid in its transmission and sustenance [5]. Both are usually transmitted by the bite of an infected tsetse fly and are more common in rural areas [6]. Detection of infection is via finding the parasite in a blood smear or in the fluid of lymph node of the suspected individual [7].

Animal African trypanosomiasis (AAT) is a serious and delabidating disease of livestock caused by protozoan parasite of the genus *Trypanosoma* and transmitted cyclically by tsetse (*Glossina*) and mechanically by other hematophagous flies such as *Tabanus, Haomatopota, Stomoxys and chrysoys* [8]. Two tsetse transmitted parasites, *T. brucei gambiense* and *T. brucei rhodesiense*, cause human African trypanosomiasis (HAT)/sleeping sickness, which affects both humans and animals [1]. The remaining tsetse-transmitted trypanosomes primarily affect animals and cause African animal trypanosomiasis (AAT). The most important species in this disease are *Trypanosoma congolense, T. vivax and T. brucei brucei.* Other species such as *T. simiae and T. godfreyi* can also cause AAT.

It is major obstacle to livestock production on the African continent as it prevents full use of land to feed the rapidly increasing population. The limitations due to tsetse and trypanosome problems continue to frustrate efforts and prevent progress in the livestock and crop production thus contributing to hunger, poverty and the suffering of entire communities in Africa [9]. This disease is therefore a serious impediment to agricultural and economic advancement in the affected areas [10]. The economic losses due to

AAT are approximated to be over 1.5 billion dollars annually with African farmers spending 35million dollars on treatment within the same time frame [11]. This very figure shows the very high economic losses for the areas infected with tsetse flies [12]. Added to the economic losses is the tsetse infestation of about 10 million km^2 of the fertile land spread across 36 countries on the Africa continents with approximately 7 million km^2 of the infected land being suitable for mixed agriculture if this disease was controlled [11]. Out of 165 million cattles found in Africa only 10 million are found within the tsetse belt due to the disease constraint and these are the lowest producing breeds [13,14]. This devastating disease vastly and directly affects the milk and meat productivity of animals, reduce the birth rate and increase the abortion rate as well as mortality rate which cumulatively affect the herd size and herd composition [12,15].

A nutraceutical is referred to as "any substance that is a food or a part of a food that provide medical or health benefits; including the prevention and treatment of diseases'' [16]. Today, researchers have identified hundreds of compounds from both plants and animals sources with functional qualities, and they continue to make new discoveries surrounding the complex benefits of phytochemicals (non-nutritive plant chemicals that have protective or disease preventive properties) in foods. In Japan, England and other countries, nutraceuticals already have become part of the dietary landscape. Due to risk of toxicity or adverse effect of drug, consumers are turning massively to food supplements to improve health where pharmaceutical fails. This resulted in a worldwide nutraceutical revolution [17,18]. Over 470 nutraceuticals and functional food products are presently available with documented health benefits [19]. Many of these new products that are being promoted to treat various diseases find their origin in the plant kingdom [19]. This is an obvious choice as many plants produce secondary compounds such as alkaloids, saponnins, terpenoids, tannins, phenols etc to protect themselves from infections and these constituents may be useful in the management of human infection. Many of the phyto-medicines are the typical examples. The old proverb "an apple a day will keep the doctor away'' is now replaced by '' a nutraceutical a day may keep the doctor away.''

Grain sorghum (*Sorghum bicolor* L. Moench;) ranks fifth in worldwide production among cereal crops after wheat, rice corn and barley [20,21]. Sorghum is used for human consumption and animal production [20] as well as industrial products such as alcohol [22]. Over 55% of sorghum grain produced in the world is used for human consumption and about 33% of grain used in animal nutrition [23]. Recent research has demonstrated that the consumption of whole grains reduces the risk of some major human diseases due to the dietary fibre and phytochemicals, which are mainly concentrated in the bran, such diseases are cardiovascular disorders, neuron degeneration, cancer, diabetes and hypercholesterolemia as well as being involved with the process of ageing [24,25]. Sorghum is a rich source of various phytochemicals including tannins, phenolic acids, anthocyanins, phytosterols and policosanols [26]. The phytochemicals have potential to significantly impact human and animal health. Sorghum grain has 95 to 98% of the nutritional value of maize; vitamin content for corn and sorghum is similar but sorghum has a higher mineral content than maize [27]. Its rich antioxidant properties makes Sorghum grain to have a lot of nutritional benefits [28]. It is higher in protein (11.5 to 16.5%) and calories than several other grains [29]. One hundred gram (100 g) of sorghum was found to contain 143 g of carbohydrate, 12 g of dietary fibre, and would provide 47% of the recommended daily value for iron based on a 2,000 calorie intake [30]. The same 100 g of sorghum contains 325 calories and has 10.8 mg of protein, 0 mg of sugar, 3.1 mg of fat, 6.0 mg of fibre and 0 mg of cholesterol. Vitamins and minerals such as: B, B_2, B_3, calcium (Ca) recommended dietary allowance (RDA) of 5.4%, potassium (K) 19% RDA, iron (Fe) 47% RDA, phosphorus (P) 55%, and sodium (Na) 0.5% RDA were found to be contained in sorghum [30].

Allium sativum, commonly known as garlic, is a species in the onion genus, *Allium.* Its close relatives include the onion, shallot, leek, chive, [31,32]. With a history of human use of over 7,000 years, garlic is native to central Asia, [33] and has long been a staple in the Mediterranean region, as well as a frequent seasoning in Asia, Africa, and Europe. A member of the *Liliaceae* family, garlic (*Allium sativum*) is a cultivated food highly regarded throughout the world. Originally from Central Asia, garlic is one of the earliest of cultivated plants [34]. In 1997, garlic was the most widely used natural supplement in US households. Garlic was also shown to be used more than twice as much as any other natural supplement [35].

That sorghum contained and in sufficient proportion daily requirement allowance (RDA) of most of the vitamins, minerals and other macromolecules required for all the physiological activities required to sustain life. Its consumption was revealed to strengthens the immune system, helps in the elimination of toxic waste from the body, increases endurance, assists in blood cells building, boost appetite, relieves diarrhoea, aids rapid recovery, stimulates free flow of blood and lowers cholesterol levels [36]. Sorghum is also reported to contain varied array of phenolic compounds such as simple phenols, hydroxybenzoic acids, hydroxycinnamic acids, flavonoids (flavanols, flavones, flavanones, isoflavones and anthocyanins), chalcones, aurones (hispidol), hydroxycoumarins, lignans, hydroxystilbenes and polyflavans (proanthocyanidins and prodeoxyanthocyanidins) [37,38]. The main thrust of this work is therefore to investigate the possible synergistic activities of the various phytochemicals from these plant samples (i.e.Sorghum and gallic) with a view to come up with a possible functional food(s) that is easily obtainable, affordable, accessible and above all commonly available in the areas mostly ravaged by this dilapidating, and neglected “orphaned” disease of the tropics.

2. MATERIALS AND METHODS

2.1 Samples Collection and Preparation

Ten kilogram (10 Kg) of red variety of *Sorghum bicolor* along with 1000 g (1 Kg) of *Allium sativum* (Garlic variety) were purchased from the Sabo/Wawa Market in Borgu Local Government Area of Niger State, Nigeria. The sorghum 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 (1000 g) 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 to obtained fine powder. Conventional rat feed was obtained from the sales point within Minna metropolis in Niger State. Graded level inclusion of the Sorghum-garlic blend at 5, 10, 15, 20, and 25% was prepared.

2.2 Trypanosomes Used

The breed of the parasite used in this work is *T. b. brucei which* affects both man and domestic animals*.* The strain of the parasites was obtained from the Nigeria Institute of Trypanosomiasis Research (NITR), Kaduna field station, Kaduna State. The parasite was maintained in our Laboratory by serial passage to healthy rats.

2.3 Experimental Animals

A total of 30 Albino Wister rats weighing between 137 – 152 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).

2.4 Feeding Experiment and Parasitaemia

From the mean of daily consumption of conventional feed determined (i.e.168g±8.6), 5% (8.4 g), 10% (16.8 g), 15% (25.2 g), 20% (33.6 g) and 25% (42 g) of the conventional feed was replaced with the sorghum-garlic blend which was administered to groups A- E respectively. Group F, were fed 12.5% of the conventional feed substituted with the pulverized sorghum only (21 g). Group G, were fed 12.5% the conventional feed substituted with of garlic only (21 g). Group H, were fed 15% of the conventional feed substituted with sorghum-garlic blend (25.2 g) for three (3) days prior to infection, Group I were fed 15% as in Group H but the feeding period prior to infection was extended to seven (7) days. Group J received 100% conventional feed and served as negative control. All the feeding experiment lasted for –21 days. In all the treatments and control, the monitoring of parasitaemia was simultaneously carried out fortnightly according to the “rapid matching” method of Herbert and Lumsden, [38]. All the animals in groups A-G were infected a day after the feeding trial commenced, while those in groups H and I were infected on the 3rd and 7th day after the feeding with the substituted sorghum- garlic has commenced. Groups J served as negative control.

The infection was carried out as thus: Blood was collected by cardiac puncture with ethylenediaminetetraacetic (EDTA) acid-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 hr by microscopic examination of blood sample taken from the tail of infected rats pre-sterilised with Methylated spirit [39].

2.5 Determination of Hematological Parameters

The haematological 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), Platelatecrit and platelete distribution weight (PDW) were determined in the group prophylactically fed at 12.5% inclusion for 7 days prior to infection in which clearance was achieved and a group fed at 25% inclusion and infected 24hrs later, using the automated haematological analyzer Sysmex kx21, as reported by Bashir et al. [40].

2.6 Statistical Analysis

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

The prophylactic benefits of food plants are being investigated for potential use as novel medicinal remedies due to the presence of pharmacologically active compounds. The result from this study clearly indicates that feeding rats with diets mixed with Garlic- Sorghum (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). If not for Nok et al. and Nose, et al. [41,42] that reported on the *in-vitro* trypanocidal effect of garlic, there has not been any previous report on the efficacy of prophylactic feeding of either the sorghum or garlic against trypanosomes, but both due to their high phenolic contents, are known to portray some strong antioxidant activities [43-46]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals [47]. An interesting observation made is the inability of the parasites to survive beyond two days *In vivo* in animals fed the mixed diet at 15% inclusion of garlic-sorghum blend seven days prior to their infection (Fig. 3) whereas, those fed for three days prior to infection had the trypanosomes inoculated into them growing normally and eventually causing the havoc and all the characteristic pathologies it is known to cause but at period a bit longer than the negative control (Fig. 2). Feeding garlic (only) and Sorghum (only) as functional foods at 12.5% inclusion level separately (Fig. 1) does not also prevent or stop the growth and the subsequent multiplication of the trypanosomes in the infected animals.

The possible reason that can be attributed to the efficacy observed when the experimental animals were prophylactically fed sorghum-garlic blended diet is, while most plants, including other cereals, are lacking 3-deoxyanthocyanidins, e.g. apigeninidins and luteolinidins, sorghum is unique in containing a relatively high level of these compounds [48,49]. Sorghum grain is rich in health promoting phytochemicals: phenolic acids, sterols, policosanols, and anthocyanins. The most abundant anthocyanins in sorghum grain are 3-deoxyanthocyanidins, e.g. apigeninidin and luteolinidin [48,50,51]. The red color of the grain's pericarp is essentially due to the presence of 3-deoxyanthocyanidins [51,52] and Tzu-Chieh, et al. [53] demonstrated the effective therapeutic effect of sorghum proanthocyanidins against human melanoma. The binding of proanthocyanidins with proteins participates in their antibacterial activity [54,55]. Proanthocyanidins have been shown to inhibit the growth of human immunodeficiency virus 1 (HIV-1), influenza virus, and herpes simplex virus by blocking their entry in the host cells [56,57]. 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 [58]. Among sorghum flavanols, the flavan-4-ols have particular therapeutic interest because of their antitumor activity [59,60,61]. Flavan-4- ols revealed strong host mediated antitumor activity, which is due to the enhancement of immune response of the host animals through the actions on tumor cells and some immunocytes [62,63]. The anti cancer activities of 3-deoxy anthocyanidins in sorghum pericarp against the HL-60 cell line, derived from a patient with acute leukemia, and HepG2 is an adherent cell line derived from liver tissues of a hepatocarcinoma patient has been well elaborated by [64]. While on one hand the green, dry "folds" in the center of the garlic clove are especially pungent. The sulfur compound allicin, produced by crushing or chewing fresh garlic, produces other sulfur compounds: ajoene, allyl polysulfides (Diallylsulphides, Diallyldisulphides and Triallyltrisulphides) and vinyldithiins. the presence of S-allylcysteine has also been observed in garlic. Despite the fact that Garlic is known for causing bad breath (halitosis) [65], as well as causing sweat to have a pungent "garlicky" smell, which is caused by allyl methyl sulfide (AMS) [66]. AMS is a volatile liquid which is absorbed into the blood during the metabolism of garlic-derived sulfur compounds; from the blood it travels to the lungs and from there to the mouth, causing bad breath; and skin, where it is exuded through skin pores [67,68]. Garlic has been used for thousands of years as a remedy for many different ailments, including intestinal disorders, flatulence, worms, respiratory infections, skin diseases, wounds, and symptoms of aging. Modern research indicates that garlic may help improve heart health in a number of different ways. It is a blood thinner that helps to lower both high blood pressure and blood triglycerides. The anti-arthritic property of garlic is due to diallyl sulphide and thiacremonone [69]. Garlic also has anti-inflammatory properties. Several population studies also show an association between an increased intake of garlic and a reduced risk of certain cancers, including colon, stomach, esophagus, pancreas, and breast cancer. [70,71]. Garlic's anti-cancer properties are due to the allyl sulphides it contains [72]. One special interesting area of research on garlic and cancer prevention involves meat cooked at high temperatures. Heterocyclic amines (HCAs) are cancer-related substances that can form when meat comes into contact with a high-temperature cooking surface (400℉/204℃ or higher). One such HCA is called PhIP (2-amino-1-methyl-6-phenylimidazopyridine). PhIP is thought to be one reason for the increased incidence of breast cancer among women who eat large quantities of meat because it is rapidly transformed into DNA-damaging compounds. Diallyl sulfide (DAS), one of the many sulfur-containing compounds in garlic, has been shown to inhibit the transformation of PhIP into carcinogens [73,74]. DAS blocks this transformation by decreasing the production of the liver enzymes (the Phase I enzymes CYP1A1, CYP1A2 and CYP1B1) that transform PhIP into activated DNA-damaging economical to produce. The future is bright, but significant investment in evaluation and feeding compounds. 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. 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; it can be seen that Anthocyanins, Pro anthocyanidins and 3-deoxyanthocyanidins contained in sorghum and the diallyl sulphide found in garlic inhibits the transformation of PhIP (a type of compound that has been associated with increased incidence of breast cancer) into carcinogen. The probable interaction of these compounds in sorghum and garlic might synergistically impair with one or more of the survival pathways of the trypanosome as observed (Fig. 3). Also, the positive immunomodulatory effect observed as a result of sustained feeding of the experimental animals (for seven days) with the sorghum-garlic (as functional foods) can clearly be seen from the Haematological parameters in (Table 1) which might be an additional attribute to the ability of the experimental animals to resist the infection by the parasites.

Currently, most antiparasitic drugs are considered orphan drugs with the main exception of antimalarials. Economic considerations of the pharmaceutical industry outweigh all others, because of the very low return of the developmental costs. Therefore, it is necessary to find alternative and cheaper ways to approach the treatment of trypanosomal disease [6]. This could be achieved by looking into the possibilities of utilising one or combination more than one functional food substances commonly available, affordable and accessible with nutraceutical properties that could serve the same purpose. Sorghum contains large quantities of phenolics and other compounds of use in human foods to prevent deterioration of health. Different phenols with varying properties exist, but relatively little effort to demonstrate the potential of these compounds in human health has been made. Of all the cereals, sorghum has the potential to be bred specifically to produce high levels of different phenols that can be easily concentrated by simple processes. These special sorghums have reasonable grain yields and agronomic characteristics that make them productive and trials to demonstrate their health promoting properties is required [75]

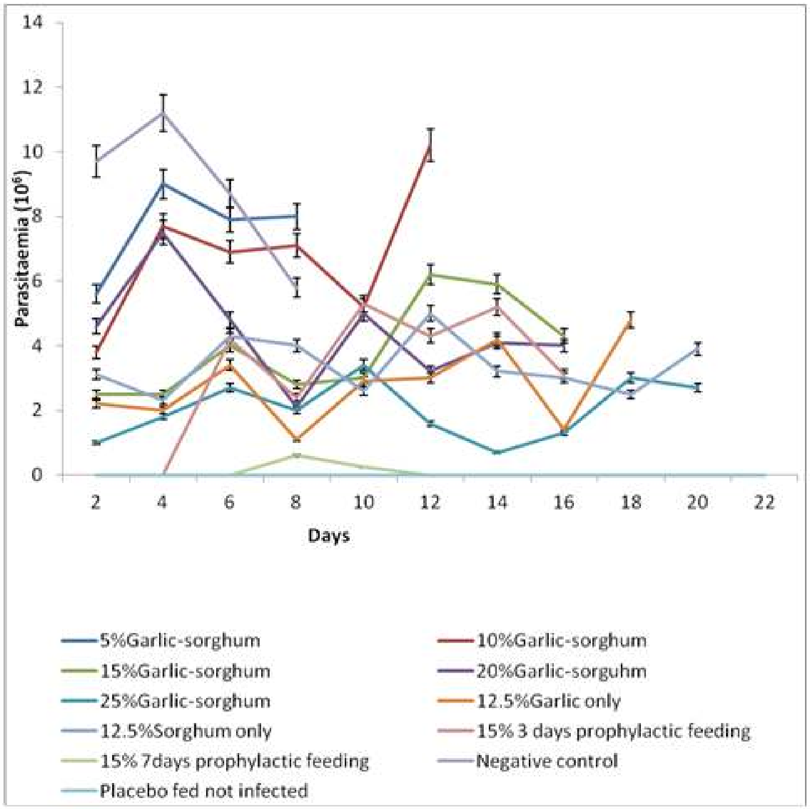

Fig. 1. Trypanocidal effect of various inclusion level of sorghum-garlic blend in the conventional feed

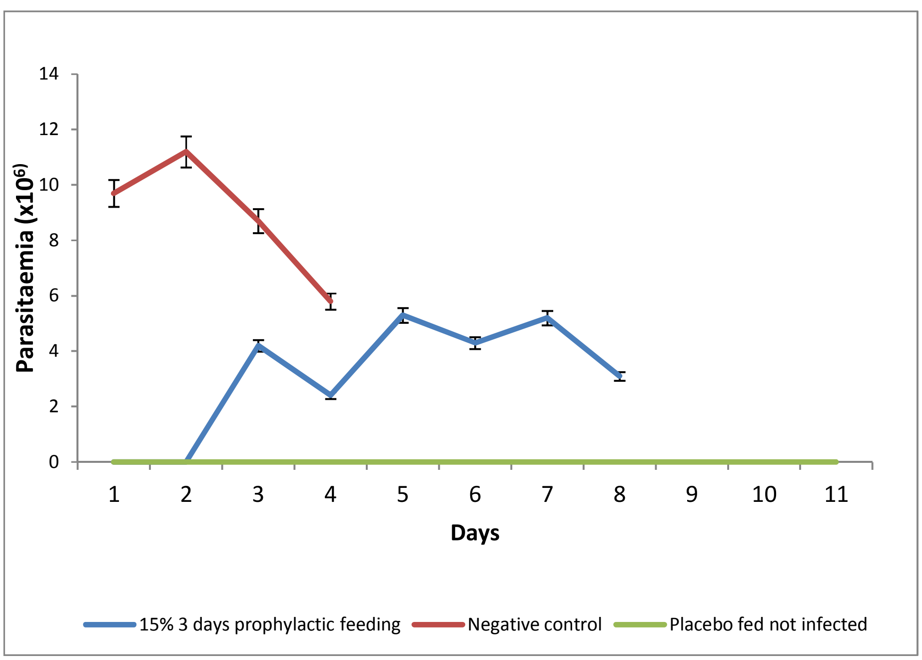

Fig. 2. Prophylactic activity of the functional food fed for three days prior to infection

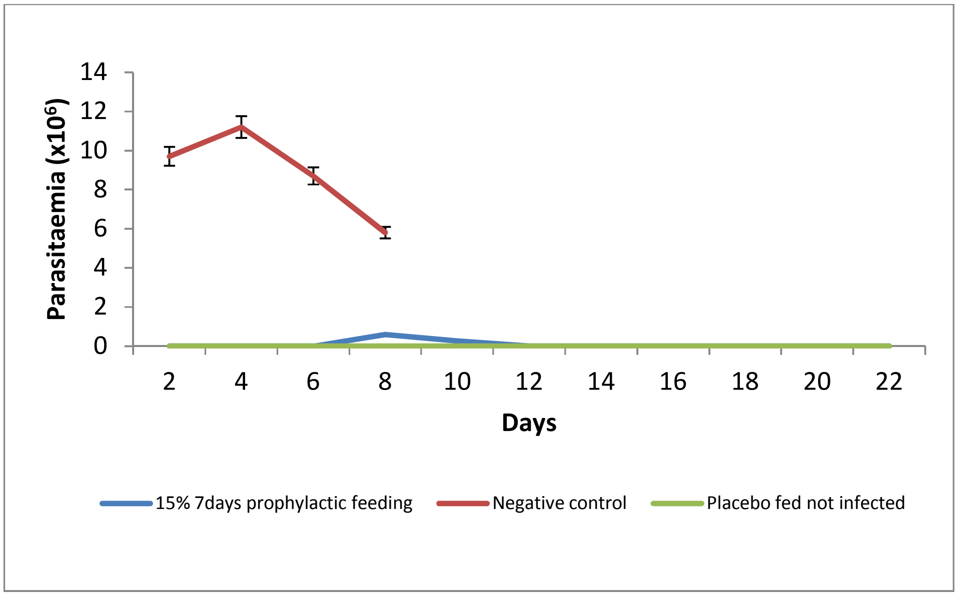

Fig. 3. Prophylactic activity of the functional food fed for seven days prior to infection

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

Parameters	Control rats (fed solely conventional feed)	Fed 12.5% inclusion 7 days prior to infection	Non prophylactic 25% inclusion
WBC (x 10^9 /L)	3.71±1.11[a]	6.58±1.18[b]	5.29±0.90[c]
Granulocytes (%)	0.80±0.70[a]	0.71±0.24[a]	2.59±0.80[b]
Lymphocytes (x 10^9 /L)	2.58±0.60[a]	4.50±0.58[b]	3.86±0.79[b]
MCTC (x10^9/L)	0.21±0.08[a]	0.12±0.005[a]	0.28±0.37[b]
RBC(x10^{12}/L)	2.69±0.10[a]	2.89±0.05[a]	0.48±0.77[b]
Hematocrite (L/L)	0.24±0.01[a]	0.22±0.00[a]	0.21±0.003[a]
Haemoglobin (g/L)	96.23±1.20[a]	94.78±1.85[a]	95.00±5.03[a]
MCH (pg)	29.96±1.59[a]	31.50±0.35[a]	29.67±1.31[a]
MCHC (g/L)	388.33±2.74[a]	434.87±3.60[b]	399.00±2.98[a]
MCV (fL)	99.23±1.20[b]	94.93±1.85[b]	73.33±0.33[a]
RCDW-cv (fL)	41.13±0.08[a]	36.23±3.16[a]	41.43±0.93[a]
RCDW-sd (fL)	18.00±0.36[a]	15.97±0.11[a]	16.00±0.36[a]
PC (× 109/L)	336.78±36.08[a]	806.65±6.83[b]	759.00±26.50[c]
MPV (fL)	9.00±0.09[a]	9.79±0.35[a]	13.01±0.30[b]
Plateletecrit (L/L)	0.64±0.15[a]	1.48±0.15[b]	1.78±0.17[b]
PDW (%)	19.46±0.41[a]	18.59±0.03[a]	26.23±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: Platelet count; Values are mean±SEM of 5 determinations. The values along the same row with different superscripts are significantly different (P = 0.05)

4. CONCLUSION

Result obtained from this studies reveals the nutraceutic effect against trypanosome infection and also haematopoietic potentials of sorghum-garlic blend when incorporated in the diet at a determined inclusion level (12.5%). This finding therefore, provide a cheap and readily available disease management alternative for those in the tse tse fly infested areas, those migrating across the tse tse infested belt or those in areas ravaged by civil unrest/wars(e.g south Sudan, Congo DR and Central Africa republic) whose environment has been re-colonised by the tse tse flies.

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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