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Ethanolic Clove Extract of *Allium Sativum* (Garlic) Caused Increased Fragility of Erythrocytes in *E Coli* Induced Sepsis of Wistar Rat

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ABSTRACT

Background: Sepsis is a major concern in intensive care Unit and its epidemiology is increasing over the years. One of the causes of sepsis include the gram negative Escherichia coli. Over the years, spices have shown to combat microbial infection., though the studies have shown that they also present certain adverse effect in the body systems. Allium sativum is one of such spices that has been of interest of study.

Objective of Study: The objective of this work is to determine the effect of ethanol extract of allium sativum on hematological parameters and osmotic fragility of red blood cells of Wistar rats during E. coli induced sepsis.

Methodology: Thirty (30) rats were used in this study. The rats were grouped into 5 groups (n=6) and were all allowed free access to food and water. Except for groups 1, the normal control group, all groups were induced with sepsis by intraperitoneal injection of E coli isolates. Successful induction was confirmed after 5 day as local signs of inflammation and sepsis such as fever and weight loss, indicative of sepsis, manifested. All groups, except group 1 and group 2, the negative control group, were treated for 14 days. Group 3, the positive control group, was treated with 400mg/kg/day of hydrochloride ciprofloxacin. Groups 4 and 5, the low and high dose groups, were treated with 200mg of extract/kg/day and 400mg of extract/kg/day respectively. After 14 days, the animals were euthanized by anaethesia using chloroform. Blood was collected by cardiac puncture and EDTA bottles. The hematological parameters were measured using automated hematological analyzer. RBC osmotic fragility test, using varying dilutions of NaCl solution and spectrophotometer was carried out. Statistical analysis of the data was done and the differences between the means of control and experiment groups were determined using ANOVA. Values of $P<0.05$ and $P<0.01$ were considered significant.

Results: Ethanol extract of garlic caused an increase in red blood cell count and hemoglobin concentration when compared to group 2 and standard drug. It only increased packed cell volume in comparison to sepsis non-treated group, Group 2. Garlic also increased osmotic fragility of RBCs. It made the RBCs more susceptible to osmotic fragility, showing impairment of membrane stability. This increased osmotic fragility by garlic could led to or even worsen hemolytic anemia. Decreases in total white blood cells and neutrophils were noted when compared to group 2. Garlic increased monocyte counts when compared with standard drug. Garlic suppressed lymphocyte count when compared to the standard drug, indicating its advantage against the immunological effect of the drug use. Garlic also increased the platelet count when compared to the negative control group but in comparison to the standard drug, the increased was significant at 200mg/kg/day treatment with garlic. **Conclusion:** Use of garlic to treat infection should not be encouraged unless with hematinics.

Keywords: Allium sativum, ciprofloxacin, sepsis, hematological parameters, Osmotic fragility, RBCs, anemia.

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INTRODUCTION

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection" (Singer *et al*., 2016). It ranks as the leading cause of death in non-cardiac intensive care units, ICU [3] and its incidence is increasing steadily. World sepsis day newsletter (2014) [32] reported 8 million annual deaths worldwide due to sepsis. In Africa, there is limited information on the epidemiology, management and outcomes of sepsis because of low awareness and limited resources [2]. The majority of cases of sepsis are due to bacterial infections [31] which tends to increase over the years. Almost all blood cells are affected by sepsis [11], especially erythrocytes [25]. Sepsis and antibiotics such as ciprofloxacin, both pose a serious adverse effect on hematological parameters basically resulting to anemia [14], [26], [17]. This adverse effect of antibiotics on blood has made patients to be placed on hematinics which are relatively expensive. Studies have shown garlic to be promising in its antibacterial effects especially against Escherichia coli infection/sepsis [14],[21], and other forms of bacterial infection [12],[21]. However, there are yet to be resolved controversies on the use of garlic to improve blood cell counts [1],[5],[7],[8],[16]. This study is design to investigate whether garlic can be an alternative in treatment of sepsis.

MATERIALS AND METHOD

Animals and Animal Grouping

A total of 30 Wistar rats, weighing within 100grams – 110 grams, were used in this study. They were acclimatized for 12 days. They were grouped randomly into five (5) groups and housed in cages under room temperature. All rats had access to rat chow and water *ad libitum*.

Preparation of Allium sativum (garlic) cloves extract and Ciprofloxacin

Fresh *Allium sativum* (garlic), sufficient for the study, were purchased from a local garden in Enugu State, Nigeria. Identification and authentication of garlic was done at the department of Plant science and biotechnology, University of Nigeria. A sample of it was kept in the herbarium with herbarium voucher specimen number UNH812a for reference purpose. Ciprofloxacin was bought from a pharmacy store; Drug Haven Pharmacy Ltd, in Enugu Metropolis in Nigeria. The drug was manufactured by Baroque Pharmaceutical PVT. Ltd, India. It was made for Miral Pharm Ltd, Nigeria. Drug number was 2BQEF254-0 with Batch Number G017103, and NAFDAC registration number B4-6438. Drug colour was Titanium Dioxide BP. Ciprofloxacin is a standard drug used in the treatment of sepsis [14],[22]. Its inhibition of bacterial gyrase and interference with DNA transcription is similar to the action of garlic [14]. It is the most commonly used Fluoroquinolone at present [10].

Preparation of ethanol extract of garlic was done in accordance with method adopted by Fowotade *et al* (2017) [8]. The garlic cloves were peeled and cut into pieces and cold extraction was carried out at room temperature (18-22°C). The whitish inner bulbs were grounded into a fine paste using a mechanical grinder. 500g of the paste was put in a standard volumetric flask and covered with about 1000 ml of 70% ethanol stoppered with cotton wool. It was allowed to stand in the dark at room temperature for 48 hours for complete extraction. The ethanol extract was filtered using pre-weighed evaporating dishes, while the residue in the flask was washed with a further 1000 ml of 80% ethanol and added to the extracts in the evaporating dishes. The filtrates were evaporated into a paste using a rotary evaporator at 40 °C. The extract was pooled together into an airtight container and stored at -4°C until required for use. In comparison with ciprofloxacin, 200mg/kg and 400mg/kg of extract were used as low and high dose. The extract was given by means of oral gavage. Extract was stored in the refrigerator at 4^0C. 400mg/ml of extract was dissolved in 100ml of distilled water to give stock solution.

Effective dose in ml = 0.5ml and 1ml for low and high dose of extract respectively.

Ciprofloxacin solution was prepared according to the method of Nouaille-Degorce *et al* (1998)[15]. Ciprofloxacin solution (10g/liter) was prepared in 0.9% NaCl solution. This gave a concentration of 10mg/ml. 400mg/kg of standard drug was used in this work [4].

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Induction of Sepsis

The method according to Oscar *et al* (2008)[18] was used to induce sepsis. Sepsis was induced in all groups except group 1(normal control). *E. coli* ATCC 25922 was grown in an intestinal tissue infusion broth. When the bacteria were in the log phase of growth, the suspension was centrifuged at 1,000 µ*g* for 15 min, the supernatant was discarded, and the bacteria were resuspended and diluted into sterile saline. All rats, except for those in group 1, were anesthetized as mentioned in the first experimental condition. The abdomen of each rat was shaved and prepared with iodine. The rats received an intraperitoneal inoculum of 1 ml of saline containing 1 X 10^8 CFU (colony forming units) of *E. coli* ATCC 25922 Isolates. Immediately after the bacterial challenge, each group of rats received intraperitoneally, an isotonic sodium chloride solution except for group 1 (normal control)

Animals were monitored for 5 days for local signs of inflammation and sepsis such as anorexia, weight loss, diarrhea, fever, and behavioral alterations indicate sepsis induction. The data for weight loss and temperature was recorded and extend to the final day of treatment to verify the antibacterial effect of *Allium sativum.* They were all observed in the experimental rats except those in group 1.

Experimental Design

Experimental field work lasted for 35 days. The doses was given by means of oral gavage for 14 days. The experimental design was as follows:

Group 1: (normal group): Normal and non-treated group

Group 2 (negative control): sepsis non-treated group

Group 3 (positive control): Sepsis treated + hydrochloride ciprofloxacin (400mg/kg/day)

Group 4: Sepsis treated + low dose of extract (200mg/kg/day)

Group 5: Sepsis treated + high dose of extract (400mg/kg/day)

Collection of Blood Sample and Determination of Erythrocyte Osmotic Fragility

The rats were anaesthetized using chloroform. Blood was collected by cardiac puncture using 5ml syringe and was stored in a 2ml EDTA tubes. The blood cell counts were measured using automated hematology analyzer.The erythrocyte osmotic fragility of rats was evaluated using the method by Oyedeji *et al* (2015)[19]. One percent (%) sodium chloride (NaCl) solution was buffered with phosphate solution, Na_2HPO4 (1.3 mg/mg) and $NaH_2PO4.2H2O$ (0.24 mg/mg). Lower dilutions of NaCl solution (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8% and 0.9%) were prepared in test tubes and the tenth test tube contained only distilled water (0.0%). The pH of the distilled water (7.0) and those of the NaCl solutions (7.4) were measured using a pH meter. Five millimeters of each concentration of NaCl was put in a test tube (9 in all) and 5 ml distilled water (0.0%) was put in the tenth tube. 0.02 ml of blood was pipetted to each test tube using a micropipette. The contents were thoroughly mixed and allowed to stand for thirty minutes at room temperature. The test tubes were then centrifuged at 3,000 rpm for ten minutes. The Optical Density (O.D.) of each supernatant solution (a measure of the degree of hemolysis) was measured with a spectrophotometer (SM23A) at a wavelength of 540 nm using a tube of distilled water as blank. The degree of hemolysis in the distilled water test tube was taken as 100% and the others were read in relation to it.

$$\text{Percentage hemolysis} = \frac{\text{O.D. of Test Solution}}{\text{O.D. of Standard Solution}} \times \frac{100}{1}$$

A cumulative erythrocyte osmotic fragility graph was obtained by plotting the mean percentage hemolysis for the five groups of rats against the concentrations of the NaCl solution.

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Statistical Analysis

Statistical analysis was performed with Statistical Package for Social Science(SPSS) version 20. Data was presented as mean ± standard error of mean (SEM). The difference between the means of control and experimental groups were determined using One Way Analysis of Variance (ANOVA). Values of $P<0.05$ and $p<0.01$ were considered statistically significant.

RESULT OF STUDY

Sepsis Induction

1) Changes in Body Temperature of Experimental Animals: There was increase in body temperature above normal range of 35.9-37.5^0 C, in all groups except for group 1. All treated groups had decreased body temperature on day 5 after sepsis induction. This showed that sepsis induction was successful (Arturo *et al.*, 2012). All the animals in group 2 (untreated group) died 5 days after commencement of treatment.

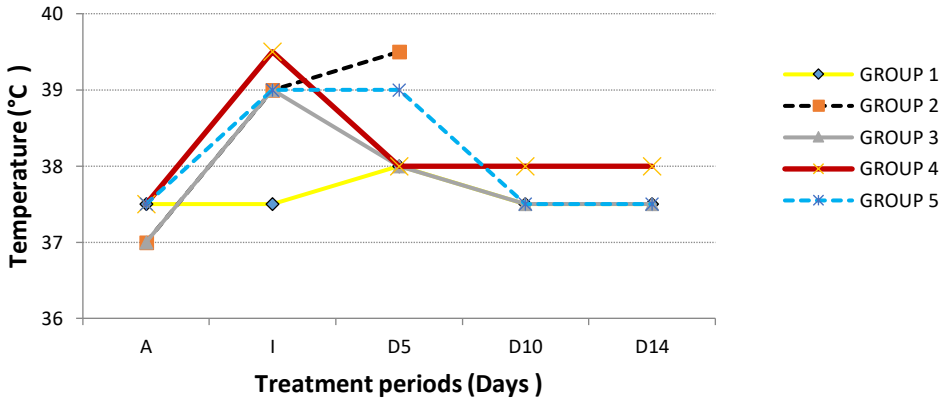

Figure 1: Changes in body temperature before and after induction. Before induction (A), 5 days after induction (I), 5 days after treatment (D5), 10 days after treatment (D10), 14 days after treatment (D14). Values are expressed as mean ±SEM, N=6.

2) *Changes in Body Weight of Experimental Rats During Treatment:* The animals in all groups, except group 1, lost weight after induction of sepsis, but regained weight following treatment with standard drug and extract. The gain in weight of group 1 was progressive from the start of the experiment up to the 14 days of treatment in treatment groups. All the animals in group 2 (untreated group) died on day 5 after induction of sepsis.

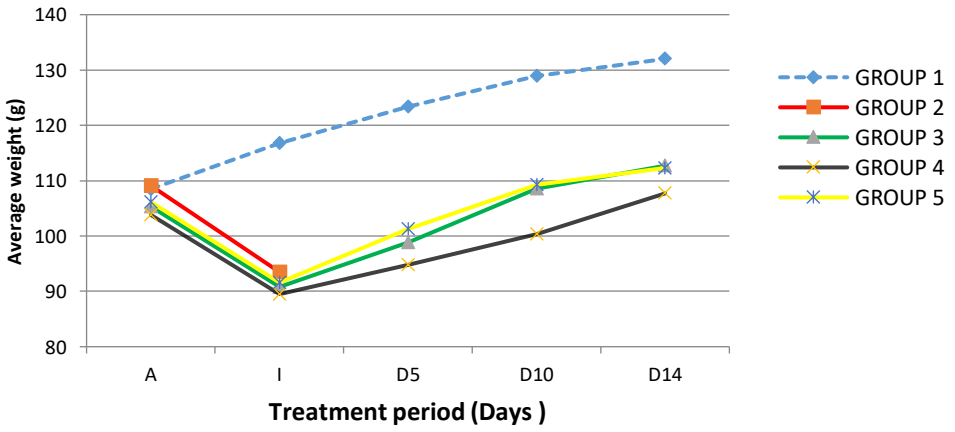

Figure 2: Changes in body weight before and after induction. before induction (A), 5 days after induction (I), 5 days after treatment (D5) , 10 days after treatment (D10), 14 days after treatment (D14). Values are expressed as mean ±SEM, N=6.

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3) *Other indication of Sepsis:* Inflammatory growth and redness were observed in all the groups induced with sepsis after 5 days. It was seen close to the points of interperitoneal injection and at the pelvic regions. However the inflammatory manifestations disappeared but not completely. The inflammatory manifestations in group 2 persisted till death. Also, food withdrawal and calmness was observed in all the groups (except group 1) at the 4th day but was more pronounced at the 5th day after induction.

Result On Hematological Parameters

1) *Result On Red Blood Cell count* :The extract and standard drug raised red blood cells counts of groups 4 and 5 significantly at P<0.01when compared to group 2 and group 3 (untreated and positive control).

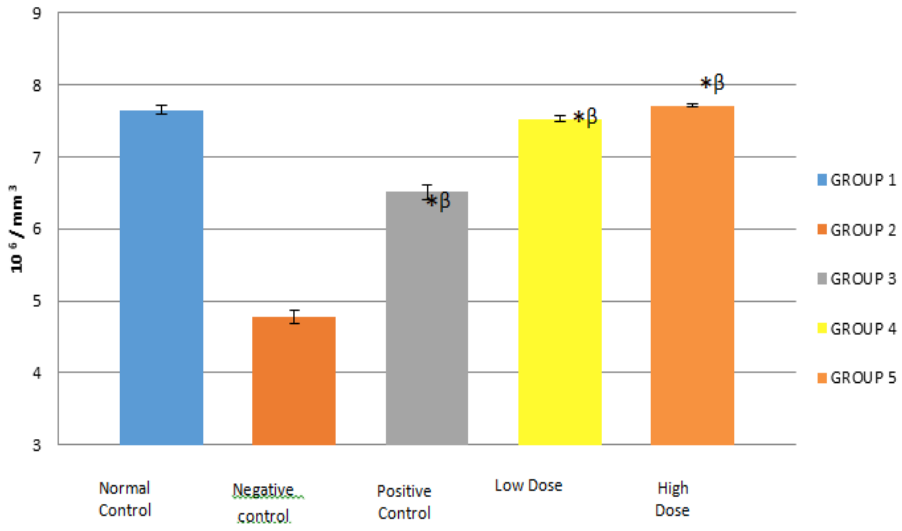

Figure 12: Red Blood Cell Count in all groups. Data expressed as mean± SEM, N=6. *P < 0.01 were considered significant when compared to group 2, βP < 0.01 compared to group 3.

2) *Result on Packed Cell Volume (PCV):* The Extract and standard drug increased packed cell volume in all treated at P<0.01. The increase was highest in group 3 (group treated with standard drug).

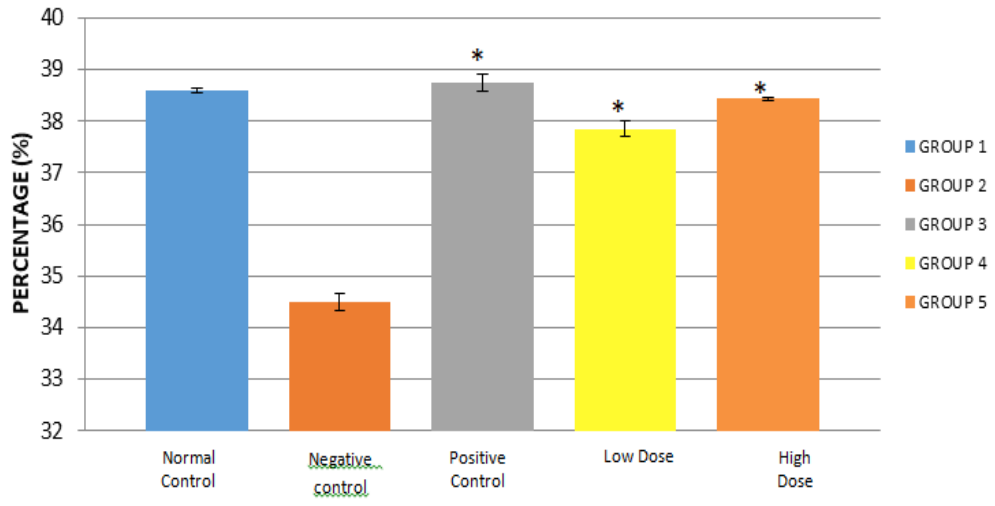

Figure 3: Packed cell volume in all groups. Data expressed as mean± SEM, N=6. * P < 0.01 was considered significant when compared to group 2.

3) *Result on Hemoglobin Concentration (HB CONC:* Extract and standard drug significantly increased the hemoglobin concentration in all treated groups at P<0.01 when compared to group 2 (untreated group). There was significant increase, at P<0.05, in other treated groups when compared to group 3 (treated with standard drug)

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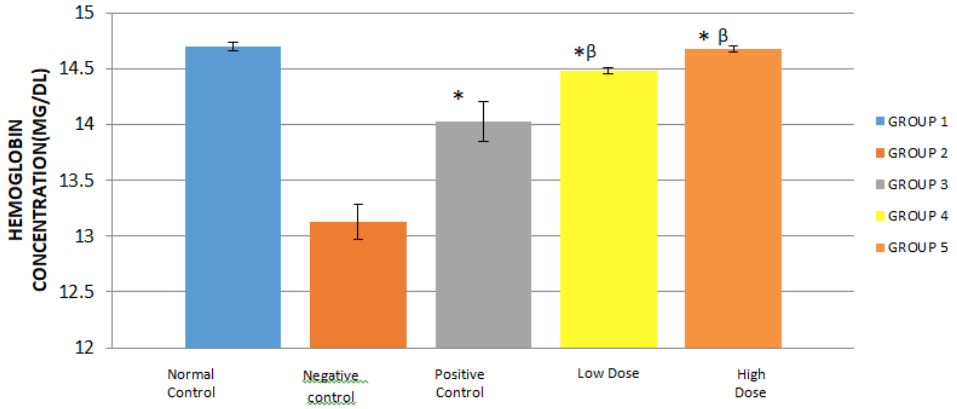


Figure 4: Hemoglobin concentration in all groups. Data expressed as mean± SEM, N=6. * P < 0.01 was considered significant when compared to group 2. β P < 0.05 when to group 3.

4) *Result on Total White Blood Cell Count (WBC:* Extract and standard drug decreased the white blood cell in all treated group when compared to the untreated group (group 2) at P<0.01. There was increase in group 4 (low dose) when compared to group 3 but it wasn't significant at both P<0.05and P<0.01.

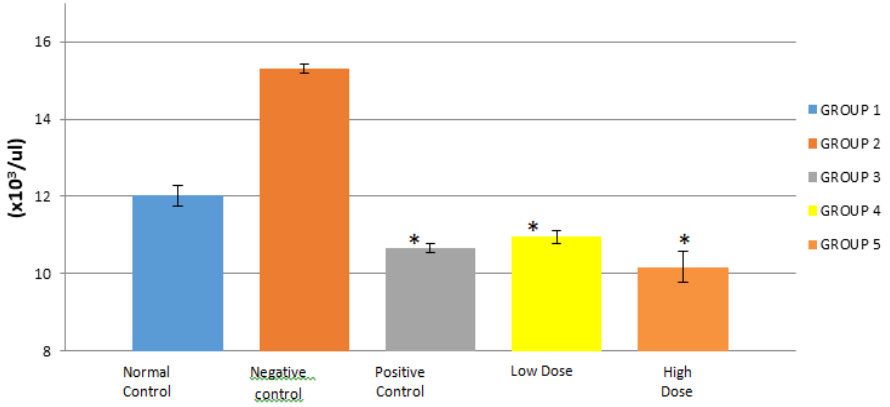
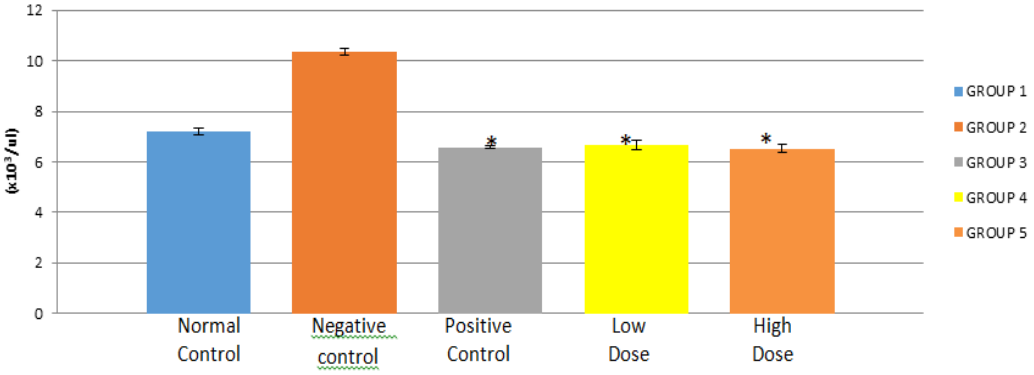


Figure 5: White blood cell count in all groups. Data expressed as mean± SEM, N=6. * p < 0. 01 was considered significant when compared to group 2

5) *Result On Neutrophil Coun:* Extract and standard drug decreased the white blood cell in all treated group when compared to the untreated group at P<0.01. There was no significant increase in other treated groups when compared to group 3 (treated with 400mg of standard drug) at both P<0.05 and P<0.01. **Figure 6: Neutrophil count in all groups**. Data expressed as mean± SEM, N=6. * p < 0.01 was significant when compared to group 2



6) *Result on Monocyte Count:* Extract and standard drug decreased the monocyte count in all treated groups, at P<0.01, when compared to group 2(untreated group). The decrease was highest in group 4 (low dose group).

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The extract increased the monocyte count at P<0.01in group 5(group treated with high dose) but decreased the monocyte count in group 4 at P<0.05, when compared to group 3 (group treated with standard drug).

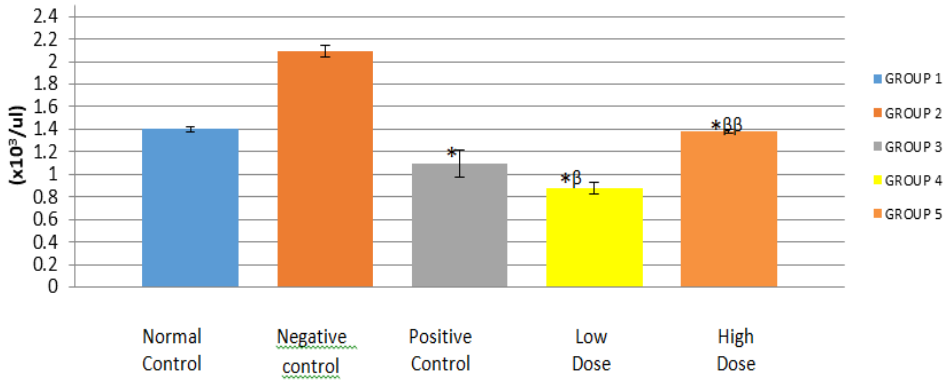

Figure 7: Monocyte count in all groups. Data expressed as mean± SEM, N=6. $^{*}P < 0.01$ is considered significant when compared to group2. $^{\beta}P < 0.05$, $^{\beta\beta}P<0.01$ when compared to group 3.

7) *Result on Lymphocyte Count:* There was significant increase at P<0.01 in group 3 when compared to group 2. There was no significant increase in lymphocyte count in group treated with extract (group 4 and 5) at both P<0.05 and P<0.01 when compared to group 2 (untreated group). There was, however, significant decrease at P<0.01in groups treated with extract when compared to group 3. The dose of extract given did not affect the decrease in group 4 and 5.

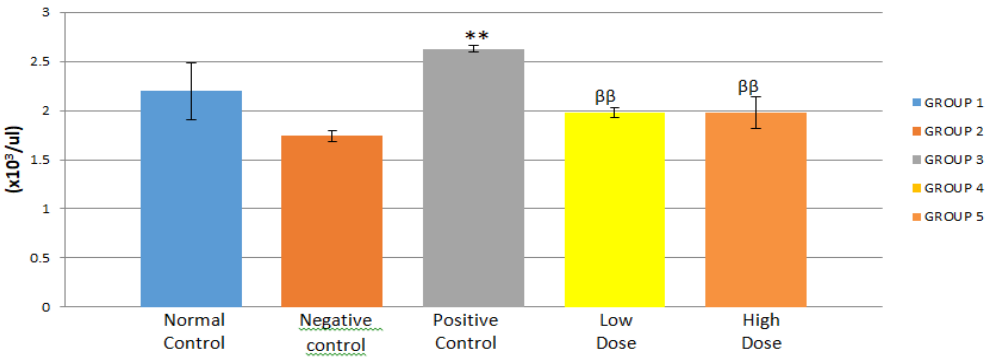

Figure 8: Lymphocyte count in all groups. Data expressed as mean± SEM, N=6. $^{*}P < 0.05$, ** $p < 0.01$ considered significant when compared to group 2. $^{\beta}P < 0.05$ and $^{\beta\beta}P<0.01$ when compared to group 3.

8) *Result on Platelets Count:* Extract and standard drug increased the platelet count in all treated groups when compared with group 2(untreated group) at p< 0.01 with lowest increase in group 4. There was decrease in group 4 (treated with 200mg of extract) when compared to group 3 (positive control, treated with standard drug) at p< 0.01.

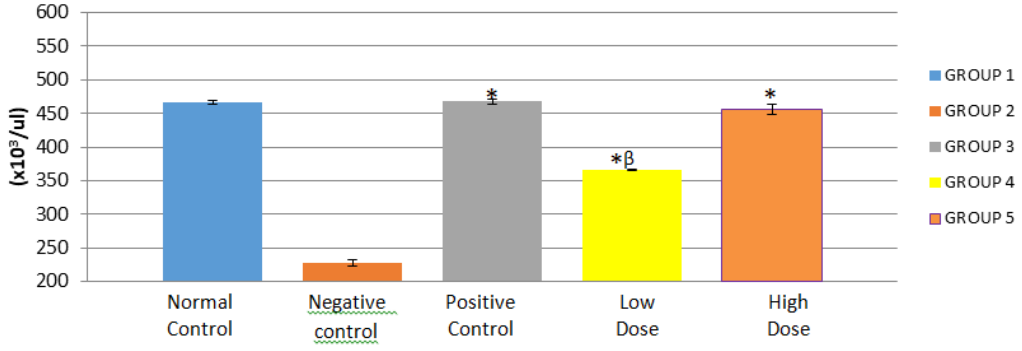

Figure 19: Platelet count in all groups. Data expressed as mean± SEM, N=6. * $p < 0.01$ were considered significant when compared to group 2. $^{\beta}P<0.01$ when compared to group 3.

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9) *Result on Osmotic Fragility of Red Blood Cells:* The osmotic fragility decreased as the concentration of NaCl increased in both treatment and control group. The percentage decrease in Osmotic fragility was greater in the control groups than in the treatment groups. The analysis showed significant increase in osmotic fragility of treated groups when compared to control groups at interval I and II. The osmotic fragility was recorded highest in group 5 when compared to group 1 at $P<0.05$. it also showed that osmotic fragility of group 2 was significantly higher in interval I (0.0-0.4% NaCl solution) but decreased in interval II (0.4-0.6% NaCl solution) and III (0.4-0.9% NaCl solution). The osmotic fragility of group 3 was significantly lower in interval I but higher in interval II and III. The percentage hemolysis decreased as the concentration increased in treatment groups. Analysis at $p<0.05$ showed an increase in osmotic fragility of groups 4 and 5 when compared to group 2 and 3.

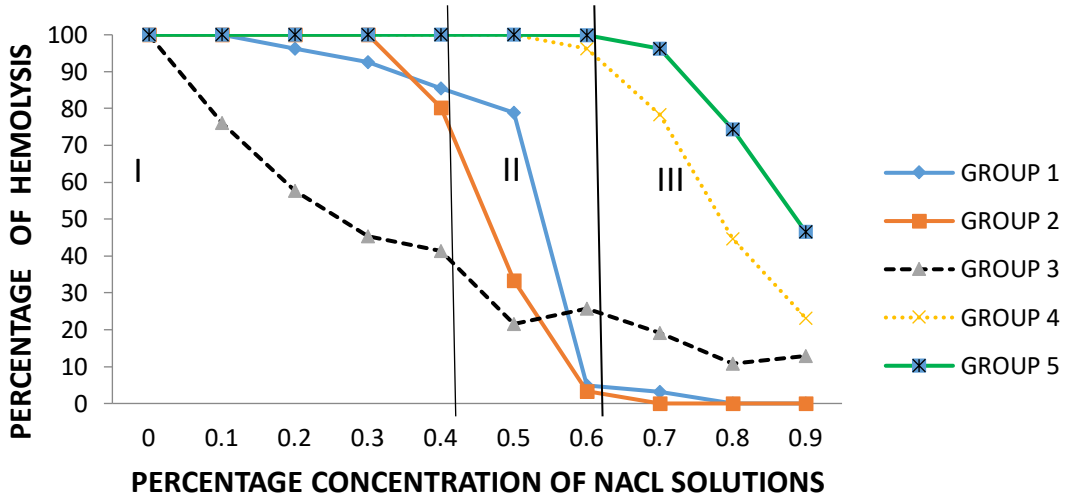

Figure 20: The effect of treatment on RBC osmotic fragility Data expressed as mean± SEM, N=6. I= Interval one (0.0 – 0.4%NaCl), II= Interval two (0.4 – 0.6%NaCl), III= interval three (0.6 – 0.9%NaCl).

DISCUSSION

Garlic is an easily available spice that is used by many homes. There are a lot of empirical studies and traditional practices that support its activity against a wide range of bacteria. This investigation was designed to evaluate its influence on blood cells against the use a standard antibiotics, ciprofloxacin

One of the signs of sepsis recovery is the lowering of body temperature to normalcy. A previous work done showed that ciprofloxacin does not increase weight [21]. The weight restoration after treatment may be due to the indirect antibacterial effect against the infection that induces loss of weight. It could also be due to ability of garlic to induce weight gain [8].

Garlic restored the red blood cell count, packed cell volume and hemoglobin concentration in septic conditions. This result agrees with some previous studies [7],[16],[29]. This is achieved possibly by increasing its production.

However, garlic decreased the leucocytosis in infection but not as much as the standard drug (ciprofloxacin). This particular result doesn't agree with the previous findings [7],[16],[29]. Enitan *et al* (2012) [7] who used crude extract of garlic. Fowotade *et* al (2017)[8] used same ethanolic extract at a dose of 250mg/kg and 500mg/kg but noted no significant increase. It is possible that the reduction in total leucocytes may be an indirect antibacterial effect of *Allium sativum* on *E coli* induced Sepsis [24] as the infectious threat has been cut off. It could also be due to method of extraction of garlic (ethanolic extraction). The ethanolic extract of garlic decreased the neutrophil count in sepsis just like the standard drug. Enitan *et al* (2012) [7] who used crude extract in their study, stated that garlic has the ability to stimulate colony stimulating factors. It is possible that the method of extraction (ethanolic extract) affected this effect of garlic on neutrophil production. However, Garlic potentiated the increase in monocyte when given with Ciprofloxacin. The ethanolic extract of garlic increased the lymphocyte count and this is in agreement with a previous study [7].

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It seems that garlic increased platelet count. This result does not agree with other related researches [9],[16],[20], who noted significant decrease. It, however, agrees with findings by Enitan *et al* (2012) who used ethanolic extract of garlic. They noted that the ability of garlic to increase platelet count has been observed to last up to 4 months of garlic supplement. This could be due to its ability to stimulate colony stimulating factors of platelets or thrombopoietin. Therefore, care should be taken in conditions that can increase platelet such as Disseminated Intravascular Coagulopathy, surgery, bone fractures and other inflammatory conditions.

The osmotic fragility of red blood cells is used as a measure of the tensile strength of the red cell membrane [4]. The data obtained from the erythrocyte osmotic fragiligraph indicated that garlic could impair the membrane stability of red blood cells even in isotonic solutions. This study agrees with the findings by Banerjee *et al* (2002)[5]. Salami *et al* (2012)[33] stated that it is due to the presence of "peroxidizable polyunsaturated fatty acids and malonyl dialdehyde" in *Allium*, and due to increased oxidation above the antioxidant capacity of red blood cells. This scientific research showed that the *A. sativum* (garlic), though may increase red blood cell production, but can cause anemia due to its effect on the membrane integrity of red blood cells during sepsis.

CONCLUSION

Both garlic and ciprofloxacin have antibacterial effect. From this study, Garlic seems to increase the red blood cell count in management of sepsis, as against ciprofloxacin, but it may also cause anemia just like the standard antibiotic drug, ciprofloxacin. It seems to cause anemia making the red blood cell more susceptible to osmotic hemolysis. It is, however, advantageous over the use of the standard drug in lymphocyte count. This means the immunological effect of the standard drug in the management of sepsis may not be experienced when sepsis is managed with garlic. In Addition, the difference in the results of this experiment and those of the previous studies could be due to the method of preparation of Extract (ethanolic extract). This study concludes that garlic causes anemia, though by a difference mechanism from the mechanism of the standard drug, and therefore should not be used together with the standard drug.

RECOMMENDATION

Garlic treatment should not be used as first line therapy (drug of choice) in sepsis management. In the combined treatment therapy, it is safer and better to be used with hematinics and at a moderate dose, if it must be used. Further studies can be carried out with other preparation of garlic aside the crude and ethanolic extract as the method of extraction may affect the potency of the spice.

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