Original Research Article

Immunomodulatory and Antibacterial Effects of Honey in Wistar rats infected with *Salmonella typhimurium.*

.

ABSTRACT

Aims: To evaluate the immunomodulatory and antibacterial effects of honey on Wistar rats infected with *Salmonella typhimurium*.

Place and Duration of Study: Research laboratory of Federal University of Technology Akure (FUTA), Ondo State, Nigeria between July 2019 to September 2019.

Methodology: A total thirty – nine (39) apparently healthy Wistar rats of three (3) rats per group were used in this study. 12 out of the rats was used to determine infectivity dose and twenty – seven (27) for infection and treatment assay. The rats were divided into nine (9) groups of 3 rats per group, all the groups were infected with *S. typhimurium* and treated for seven (7) days with honey, augmentin and oral rehydration salt (ORS) except group 1 that was infected and not treated and group 9, not infected, not treated. The blood samples of all the rats was collected after treatment to study the effect of honey on the haematological parameters of the rats.

Results: All the honey samples used exerted growth inhibitory activity on Salmonella typhimurium but honey from FUNAAB worked best both in the *in-vivo and in-vitro* studies. The rats that were infected with the *S. typhimurium* and treated with 2ml and 3ml honey gave a good therapeutic potential in combating diarrhoea in the animals. Also, in these group of rats, honey caused an increase in the PCV, RBC, HB and lymphocytes which displays honey to be a good immunostimulator and immunomodulator.

Conclusion: This honey has also exerted antibacterial, haematinic and immunomodulatory potentials when rats infected with *S. typhimurium* These findings therefore could be exploited in the treatment of diarrhoeal diseases caused by this bacterium.

Keywords: [S. typhimurium, Wistar rats, Honey, Augmentin, Oral rehydration salt (ORS), Immnuomodulation]

1. INTRODUCTION

Salmonella typhimurium are Gram-negative, flagellated, aerobic (oxygen-consuming) bacteria that are the major cause of human salmonellosis [1], a type of gastroenteritis, or inflammation of the intestine [2]. *S. typhimurium* is also a frequent cause of acute, self-limiting food borne diarrhea, it is spread primarily by contaminated food and drink, but it can come in contact with a human via direct contact with an infected animal or pet [1]. *Salmonella typhimurium* induces a systemic infection in rats, So *S. typhimurium*-infected rats have been extensively used as models for the understanding of the immunological and antibacterial effect of honey. Diarrhoeal diseases are among the leading causes of morbidity and mortality in young children in developing countries [3] It is characterized by frequent, loose and watery stool which may result in dehydration and in severe cases, death. Each year , an estimated 2.5 billion cases of diarrhoea occur among the children under five years of age, and estimates suggest that overall incidence has remained relatively stable over the past two decades. When this illness is as a result of *S. typhimurium* infections when antibiotics therapy may be required, the problem of antibiotics resistance is also a serious problem because the problem of antibiotics resistance is also a serious problem because almost all known strains of *S. typhimurium* have developed resistant to most of the commonly employed antibiotics Also, some of these antibiotics can induce diarrhoea known as “antibiotic induced diarrhoea”[4]. Therefore, it becomes imperative to search for alternatives to conventional antibiotics to treat this disease. In most ancient cultures, honey has been used for both nutritional and medical purposes. The belief that honey is a nutrient, a drug and an ointment has been carried into our days, and thus, an alternative medicine branch, called ‘‘apitherapy’’, has been developed in recent years, offering treatments based on honey and other bee products against many diseases including bacterial infections. Honey has been reported to have immunomodulatory and antibacterial activity on bacteria found in wounds [5], responsible for food spoilage[6], common diarrhoeagenic bacteria such as *S. typhimurium* [7] and many other bacterial species.

2. MATERIAL AND METHODS

2.1 Location and Duration of the Research

The research was carried out in the Graduate Research Laboratory of Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria between July to September, 2019.

2.2 Collection of Honey Samples

Honey samples were collected from ten (12) different locations in Nigeria; Emure – Ile and Afo – Akoko, Ikakuma – Akoko, Akure in Ondo State, Enugu, Enugu State, Ibadan, Oyo State, Ikere- Ekiti, Ekiti State, Lagos, Lagos State, Nasarawa, Nasarawa State, FUNAAB, Abeokuta Ogun State, Zamfara, Zamfara State and Iree, Osun State. Table 1 shows the location and the floral source of the honey samples used.

1

2 **Table 1. Honey samples from different locations in Nigeria**

S/N	Location	Floral source
1	Emure – Ile, Ondo State (Roadside)	Wildflower Honey
2	Ikere- Ekiti, Ekiti State	Wildflower Honey
3	Nasarawa, Nasarawa State	Wildflower Honey
4	Ibadan, Oyo State	Wildflower Honey
5	Afo, Ondo State	Wildflower honey
6	Ire, Osun State	Bitter leaf
7	FUNAAB, Abeokuta, Ogun State	Wildflower Honey
8	Enugu, Enugu State (Cinomis Honey)	Wildflower Honey
9	Lagos, Lagos State (Kaybeck Honey)	Wildflower Honey
10	Zamfara, Zamfara State (A & Shine Honey)	Wildflower Honey
11	Ikakuma – Akoko, Ondo State	Wildflower Honey
12	Akure, Ondo State	Wildflower Honey

2.3 Test Organism

The test organism used was *Salmonella typhimurium*

2.3.1 Isolation and identification of the test organism

S. typhimurium was isolated from the faeces of poultry droppings, the stool sample was serially diluted in sterile distilled water using the method of [8].The dilutions were plated on Salmonella Shigella agar to isolate the bacteria and was identified based on morphological and biochemical characteristics according to the method of [9].

2.4 Antibacterial assay

The antibacterial activities of all the honey samples (unpasteurised and pasteurised) on the *S. typhimurium* was done using agar well diffusion method according to [10].

2.5 Experimental animals

A total of 39 female Wistar rats of 60-90g were used for the study. The animals were purchased at Animal Production and Health Dept of Federal University of Technology Akure, Ondo State. They were brought to animal house of Microbiology department, FUTA and acclimatized for 7days before the commencement of this work. The animals were fed with broiler starter and clean water twice daily.

2.6 Experimental animals for infectivity dose

A total of 12 rats female apparently Wistar rats of 60-90g was used to determine infectivity dose. The rats were divided into four groups of 3 rats per cage.

2.6 Determination of infectivity dose (ID) of *Salmonella typhimurium*

This was done using standard method described by [11].

A colony of S. typhimurium of 24hrs old was inoculated into 100ml of Nutrient agar, incubated at 37^{o}C for 18 – 24hrs. The cells were harvested by centrifuging at 3000rpm for 15 minutes. The supernatant was decanted and 10ml of sterile normal saline was poured into the tube and was further centrifuged to wash the cells, this was done three times. Serial dilution was carried on the harvested cells. 1ml was taken from each of the different concentrations already prepared to infect the experimental animals. The dilution that produced the symptoms of illness in all of the animals was taken as the infectivity dose (ID) of the organism.

2.7 Experimental design

A total of 27 female apparently healthy Wistar rats were assigned into nine (9) treatment groups designated as 1 – 9. i.e. 3 rats per cage. Rats in group 1 were infected with the ID of *S. typhimurium* and not treated, group 2 were infected and treated with 1ml raw honey 12hourly, group 3 infected and treated with 2ml raw honey 12hourly, group 4 infected and treated with 3ml raw honey 12hourly, group 5 infected and treated with 0.5ml Augmentin (30mg/kg/day) 12hourly, group 6 infected and administered honey – ORS 12hourly, group 7 infected and administered 1ml commercial ORS 12hourly, group 8 infected and administered 1ml homemade ORS 12hourly and group 9 not infected, not treated (control group).

2.8 Infection of rats with *S. typhimurium*

The infection of the animals was done using the infectivity dose of the organism by orogastrically dosing them according to the method of [11]. The infectivity dose used in this study was calculated to be 1.5 x10^{8} cfu/ml.

2.9 Treatment of infected rats

Treatment begins 24hours after which infection has set in, specific volume of honey, augmentin, honey –ORS, ORS both commercial and home made variant were administered to the infected rats for 7 days according to [12].

2.10 Isolation, identification and enumeration of *S. typhimurium* in the faeces of infected rats

1g of faeces of the infected rats were aseptically collected, serial dilution was done on them and plated on salmonella shigella agar in order to isolate the *Salmonella typhimurium* present in the rats and monitor their bacterial count through out the experiment.[13].

2.11 Weighing of Animals

The weight of the animals were taken throughout the pre and post ingestion period using the method of [14].

2.12 Haematological Assay

The blood of apparently healthy Wistar albino rats was collected weekly into EDTA bottles after which the Packed Cell Volume (PCV), Haemoglobin (HB), Red blood Cell (RBC), White Blood Cell (WBC) and differential leukocytes counts of the collected blood samples were evaluated according to the method described by [16]**.**

2.13 Statistical Analysis

All experiments were done in triplicates, Mean, Standard deviation were calculated for all data using Descriptive Statistics and difference between means was determined by Duncan's New Multiple Range Test at $p \leq .05$.

3. RESULTS AND DISCUSSION

Almost all the honey samples used in this study inhibited the growth of *S. typhimurium*, the antibacterial activity exerted by honey on the test organism in this study is in agreement with the work carried out by [7] that pure honey has bactericidal activity against *Salmonella* species. There was a great reduction in the antibacterial activities of pasteurised honey when compared with the unpasteurised ones, this is in agreement with the work of [16] and [17] that reported the loss of antibacterial activity on exposure of honey to heat at 80^{o}C for 10mins or 56^{o}C for 30mins. Honey from FUNAAB was observed to have the highest antibacterial activity when compared with the control (augmentin) used in this study. (Fig.1).

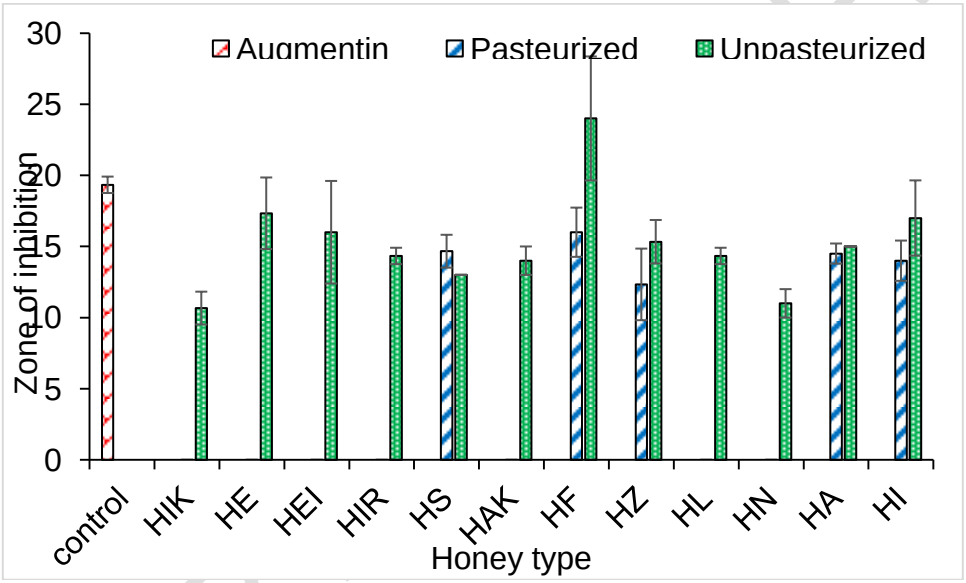


Fig. 1: Comparison among the antibacterial activities of unpasteurised and pasteurised honey samples on *S. typhimurium* isolated from poultry droppings.
Key: HIA = Honey from Ikakuma- Akoko, HIK = Honey from Ikere – Ekiti, HN = Honey from Nasarawa, HI = Honey from Ibadan, HA = Honey from Afo – Akoko, HIR = Honey from Iree, HF = Honey from FUNAAB, HE = Honey from Enugu, HL = Honey from Lagos, HZ = Honey from Zamfara, HS = Sunshine honey and HEI = Honey from Emure – Ile.

All the rats infected with this bacterium and treated with honey recovered by the 3rd day while those ones that was administered honey – ORS and the ones with homemade ORS recovered by day 4 and those treated with augmentin and those administered commercial ORS recovered by the 5th day. those that were infected but not started showing signs of recovery by the 6th day, this could be that acute diarrhoea is self limiting according to [18]. The mean recovery times of rats infected with *Salmonella typhimurium* and treated with honey

–ORS was significantly reduced when compared with infected and not treated group, this is agreement with the work of [19] (Table 2). There was no evidence of *S. typhimurium* in the faeces of rats treated with 1ml, 2ml, 3ml of honey and administered 1ml of honey –ORS 12hourly for 7 days. **(**Fig. 2)**.**

Table 2: Physical observations of the Wistar rats during infection with *Salmonella typhimurium* and treatment

Group of rats	Treatment	(Days) 1	2	Interval 3	4	5	6	7
1	Infected and not treated with honey	RA, EL, UF,PM,SS	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	A, EW, FS, NM, SF	A, EW, FS, NM, SF
2	Infected and treated with 1ml honey (12hourly)	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	A, EW, FS, NM	A, EW, FS, NM	A, EW, FS, NM	A, EW, FS, NM	A, EW, FS, NM
3	Infected and treated with 2ml honey (12hourly)	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	A, EL, UF, PM, SS	A, EL, FS, NM, SF	A, EL, FS, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF
4	Infected and treated with 3ml honey (12hourly)	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	A, EL, UF, PM, SS	A, EL, FS, NM, SF	A, EL, UF, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF
5	Infected and treated with 0.5ml Augmentin (12hourly)	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	A, EW, FS, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF
6	Infected and treated with 1ml Honey - ORS (12hourly)	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	A, EW, FS, NM, SF	A, EW, FS, NM	A, EW, FS, NM, SF	A, EW, FS, NM, SF
7	Infected and treated with 1ml ORS (12hourly)	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	RA, EL, UF, PM, SS	RA, EL, UF, *PM, SS	A, EW, FS, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF
8	Infected and treated with	RA, EL, UF, PM,	RA, EL, UF,	RA, EL, UF, PM,	A, EW, FS, *PM,	A, EW, FS, NM,	A, EW, FS, NM,	A, EW, FS, NM,

	1ml homemade (12hourly)	SS	PM, SS	SS	SS	SF	SF	SF
9	Not infected and not treated	A, EW, FS, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF	A, EW, FS, NM, SF

KEY: A = Activeness, RA = Reduced activity, EL = Eating little, EW = Eating well, UF = informed stool, FS = Formed stool, SF = Smooth fur, SS = Scattered fur, PM = Presence of mucous, *PM = High presence of mucous, NM = No mucous

There was no evidence of *Salmonella typhimurium* in the faeces of rats treated with 1ml, 2ml, 3ml of honey and administered 1ml of honey –ORS 12hourly for 7 days. (Fig. 2).

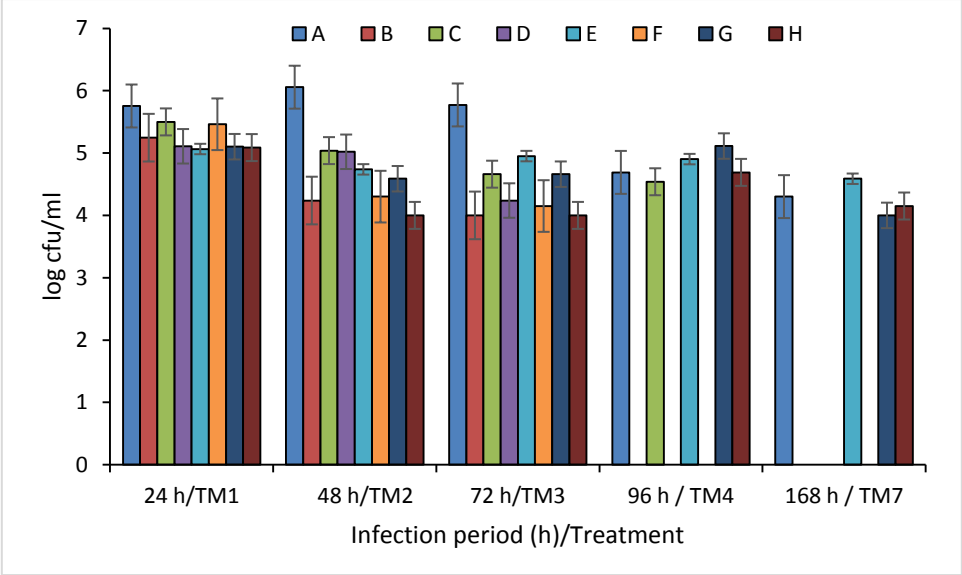
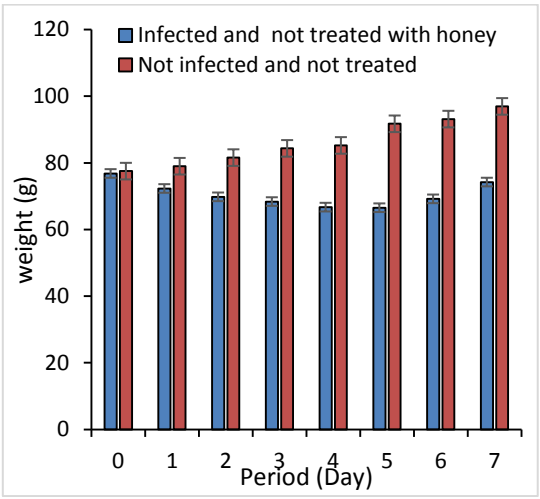


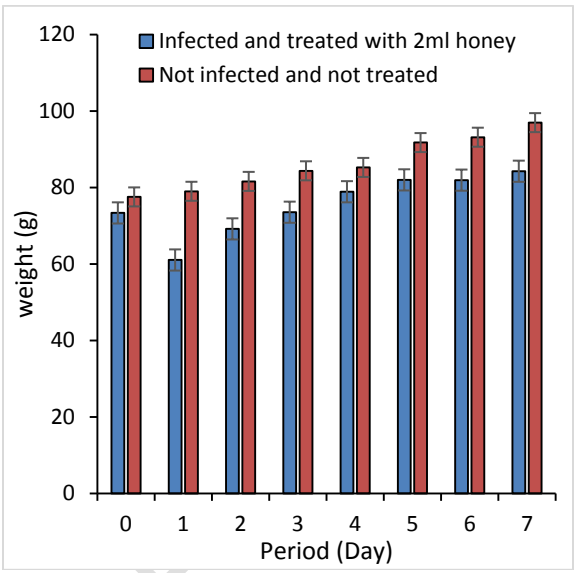
Fig. 2: Total counts of *S. typhimurium* in the faeces of Wistar faeces after infection and treatment

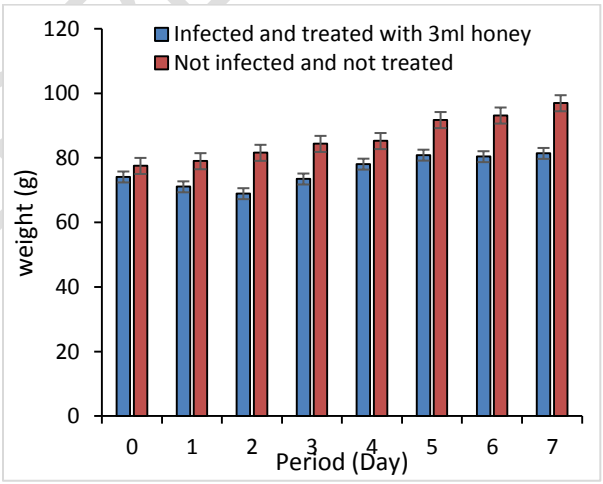
KEY: A = Infected and not treated, B = Infected and treated with 1ml honey, C = Infected and treated with 2ml honey, D = Infected and treated with 3ml honey, E = Infected and treated with 0.5ml augmentin, F = Infected and treated with 1ml honey – ORS, G = Infected and treated with 1ml commercial ORS, H = Infected and treated with 1ml homemade ORS, TM1 = Treatment day 1, TM2 = Treatment day 2. TM3 = Treatment day 3, TM4 = Treatment day 4 and TM7 = Treatment after day 7.

The observation that the infected and not treated rats did not gain back their body weight throughout the duration of the research is in agreement with the work of [14]. (Fig. 3)..

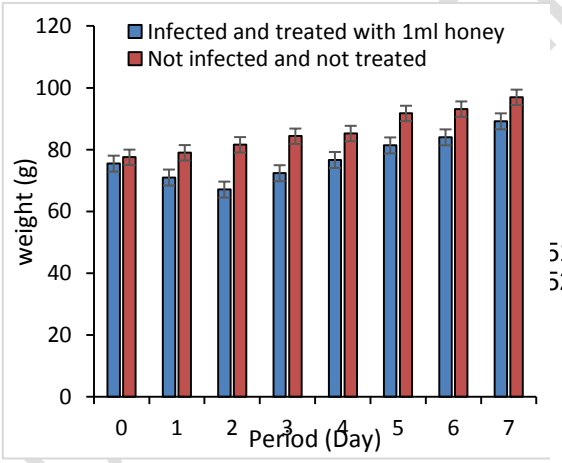

Infected and not treated with honey
Not infected and not treated
weight (g)
Period (Day)

A


Infected and treated with 2ml honey
Not infected and not treated
weight (g)
Period (Day)

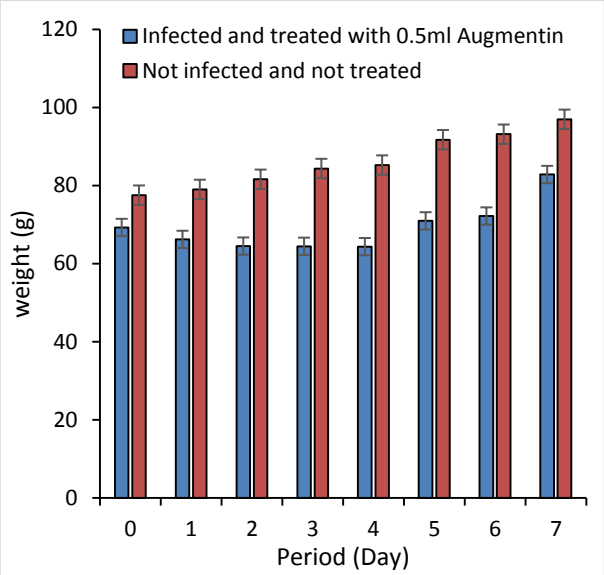

Infected and treated with 3ml honey
Not infected and not treated
weight (g)
Period (Day)

D


Infected and treated with 1ml honey
Not infected and not treated
weight (g)
Period (Day)

B

C



E

Fig, 3A –E : Average weight of Wistar rats before infection with *S. typhimurium,* at the onset of infection and after treatment with honey and augmentin.

Infection of rats in group 1 that were not treated caused a decrease in their PCV, HB and RBC, their neutrophils was so high, showing a sign of infection, there was no significant difference in the PCV, WBC of the group of rats treated with 2ml, 3ml of honey and the group not infected, not treated (control) (Table 3a and 3b). **A**dministration of honey to apparently healthy rats (control) and the groups infected with *S. typhimurium* and treated with honey caused a significant ($p<.05$) increase in the PCV and lymphocytes of the rats, this shows that the honey has haematinic and immunomodulatory potentials. (Table 3a and 3b).

Table 3a: Effect of honey on the haematological parameters of Wistar rats infected with *S. typhimurium*

Group	PCV (%)	HB (g/L)	WBC (10^9/L)	RBC (10^{12}g/L
1	32.50 ± 4.95^{b}	10.85 ± 1.66^{b}	12.70 ± 0.28^{a}	3.00 ± 0.82^{b}
2	40.00 ± 2.83^{ab}	13.50 ± 0.71^{ab}	11.70 ± 0.28^{ab}	3.20 ± 0.25^{ab}
3	42.50 ± 2.12^{a}	14.14 ± 0.66^{a}	8.77 ± 0.49^{c}	4.04 ± 0.87^{ab}
4	43.00 ± 4.24^{a}	14.30 ± 1.36^{a}	8.94 ± 0.42^{c}	4.29 ± 0.07^{a}
5	40.33 ± 4.51^{ab}	13.50 ± 1.51^{ab}	10.86 ± 0.17^{abc}	$4.06 \pm 0.3.00^{ab}$
6	35.50 ± 0.71^{ab}	11.90 ± 0.28^{ab}	10.88 ± 0.05^{abc}	3.66 ± 0.03^{ab}
7	39.67 ± 4.51^{ab}	13.19 ± 1.56^{ab}	9.56 ± 1.92^{bc}	4.07 ± 0.34^{ab}
8	35.00 ± 6.00^{ab}	11.64 ± 2.00^{ab}	9.83 ± 1.47^{bc}	3.66 ± 0.53^{ab}
9	42.33 ± 2.08^{a}	13.66 ± 0.35^{ab}	8.82 ± 1.06^{c}	4.07 ± 0.58^{ab}

Table 3b: Effect of honey on the haematological parameters of Wistar rats infected with *S. typhimurium* contd

Group	Lymphocytes (%)	Neutrophils (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
1	31.50 ± 0.71^{f}	64.50 ± 0.71^{a}	3.00 ± 0.00^{a}	1.50 ± 0.71^{a}	1.00 ± 0.00^{a}
2	37.50 ± 0.71^{bc}	61.00 ± 1.41^{ab}	2.50 ± 0.71^{a}	2.00 ± 1.41^{a}	1.00 ± 0.00^{a}
3	40.00 ± 2.83^{ab}	56.50 ± 0.71^{b}	1.50 ± 0.71^{a}	1.00 ± 0.00^{a}	0.00 ± 0.00
4	40.50 ± 0.71^{a}	57.00 ± 1.41^{b}	1.50 ± 0.71^{a}	1.00 ± 0.00^{a}	0.00 ± 0.00
5	39.33 ± 150^{abc}	56.00 ± 4.00^{b}	1.67 ± 1.15^{a}	1.00 ± 0.00^{a}	0.00 ± 0.00
6	37.00 ± 1.41^{cd}	60.00 ± 2.83^{ab}	2.00 ± 1.41^{a}	1.50 ± 0.71^{a}	0.00 ± 0.00
7	32.67 ± 1.15^{ef}	60.33 ± 3.79^{ab}	2.00 ± 1.00^{a}	1.67 ± 0.58^{a}	0.00 ± 0.00
8	34.67 ± 0.58^{de}	60.67 ± 1.15^{ab}	2.33 ± 0.58^{a}	1.67 ± 0.58^{a}	1.00 ± 0.00^{a}
9	40.00 ± 1.00^{ab}	57.33 ± 1.15^{b}	1.33 ± 0.58^{a}	1.33 ± 0.58^{a}	0.00 ± 0.00

4. CONCLUSION

This study has shown that the antibacterial activity of honey sample from FUNAAB (HF) against *S. typhimurium* as the most effective in inhibiting the growth of the organism. This honey has also exerted antibacterial, haematinic and immunomodulatory potentials when rats infected with *S. typhimurium* These findings therefore could be exploited in the treatment of diarrhoeal diseases caused by this bacterium.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

CONSENT

It is not applicable

ETHICAL APPROVAL

As per international standard written ethical approval has been collected and preserved by the author(s).

REFERENCES

1. Torpdahl M, Tsai-Ling L, Shiu-Yun L, Ishien L, Sung-Hsi W, and Chien-Shun C. "Human Isolates of *Salmonella enterica* Serovar *Typhimurium* from Taiwan displayed significantly higher levels of Antimicrobial Resistance than Those from Denmark." International Journal of Food Microbiology. 2013; 69-75.

2. Black JG. Microbiology*:* Principles and Explorations. 2008; 1
   Boschi-Pinto C, Velebit L and Shibuya K. Estimating child mortality due to diarrhoea in developing countries. Bulletin of WHO 2008; 86 (9): 710-717.

3. Chen C, Campbell LT, Blair SE. and Carter DC.. The effect of standard heat and filtration processing procedures on antimicrobial activity and hydrogen peroxide levels in honey. Frontier*s* Microbiology. 2012; 3, 265, 1-8.

4. Cheesbrough M. Medical Laboratory Manual for Tropical Countries. VOL. II. Microbiology. 2006; 479. University of Cambridge press. Great Britain.

5. Basualdo, C., Sgroy, V., Finola, M.S., and Junm, M (2007). Comparison of the antibacterial activities of honey from different provenance against bacterial usually isolated from skin wounds. *Veterinary Microbiology*. 127: 375-381.

6. Lusby PE, Coombes AL and Wilkinson JM. Bbactericidal activity of different honeys against pathogenic bacteria. Archieve Medical Resources. 2005; 36:464-467.

7. Adebolu TT. Effects of natural honey on local isolates of diarrhoea causing bacteria in Southwestern, Nigeria. African Journal of Biotechnology. 2005; 4(10):1172 - 1174.

8. Boateng J and Diunase KN. Comparing the antibacterial and functional properties of Cameroonian and manuka honey for potential wound healing. Molecules, MDPI Journals 2015; (20)9, 16068 - 16084.

9. Rozanska Hanna. Microbiological Quality of Polish Honey. National Veterinary Research Institute. Pulway. 2011; 55, 443 – 445.

10. Moussa, A, Noureddine, D, Saad A and Abdelmalek M. influence of temperaure on the inhibitory potency of eucalyptus honey against Candida albicans. A*sian* Pacific Journal of tropical disease 2012. S567-S570. Adebolu TT, Adeoye OO, Oyetayo VO. Effect of garlic (*Allium sativum*) on *Salmonella typhi* infection, gastrointestinal flora and haematological parameters of albino rats. African Journal of Biotechnology. 2011; *1*0 (35):6804-6808.

11. Adebolu TT, Adeoye OO, Oyetayo VO. Effect of garlic (*ALLIUM SATIVUM*) ON *Salmonella typhi* infection, gastrointestinal flora and haematological parameters of albino rats. African Journal of Biotechnology. 2011; *1*0 (35):6804-6808.

12. Oladunmoye MK. (2007). The Imunostimulatory effect of ethanolic extract of C*assia alata* on immune system of albino rats dosed with *Staphylococcus aureus* (ncib 8588). Journal of Pharmacology and Toxicology. 2007; 2(2): 200-204.

13. Fawole MO, and Oso BA. Characterization of Bacteria: Laboratory Manual of Microbiology. 4th EDN., Spectrum Book Ltd, Ibadan Nigeria 2004; 24-33.

14. Momoh AO, Adebolu, TT and Ogundare AO. Therapeutic effects of beniseed extracts and fermented liquor in treating diarrhoea in albino rats infected with *Bacillus cereus.* Global advanced research journal of medicinal plants. 2012; 1 (1): 007- 012.

15. Baker FJ and Silverstone RE. Medical Laboratory Science. 8TH EDITION. Chris Publisher, Washington D.C., USA. 2006; 447.

16. Chen, C., Campbell, L.T., Blair, S.E. and Carter, D.C. (2012). The effect of standard heat and filtration processing procedures on antimicrobial activity and hydrogen peroxide levels in honey. *Front Microbiol*., 3, 265, 1-8.

17. Hasan SH. Effect of storage and processing temperatures on honey quality. Journal of Babylon University/Pure and Applied Sciences, 2013*;* 6(21), 2244-2253.

18. Steven MD, Edward J, Hoffenberg MD. Imported Infectious Diseases. Acute Diarrhoea in Berman's Pediatric Decision Making. 2014; 1. 5th edition.

19. Beretta G, Gelmini F, Lodi V, Piazzalunga A, Facino RM. Profile of nitric oxide (no) metabolites (nitrate, nitrite and n-nitroso groups) in Honeys of different botanical origins: nitrate accumulation as index of origin, quality and of therapeutic opportunities. Journal of Pharmaceutical and Biomedical Analysis*.* 2010; 53 (3): 343-349.