

Assessment of the Pharmacological Single and Combined In-vivo Drug Effects on Albino-Mice in University of Jos Experimental Animals

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Abstracts

Background: The development of in-vivo anti-malarial and anti-typhoid drug has become an essential tool in research to study different drug effects, especially assessing their pharmacological effects on parasites and bacteria. Study drug effects on *Plasmodium* and *Salmonella*, specific stages at which drug activity and suppression are most effective, improves the development of new drugs and vaccines production against Malaria and Typhoid.

Methodology: A simple random sampling technique utilizing modified Peter's 4-day suppressive in-vivo culture method was used on Albino Mice, to evaluate drug effects and assess their pharmacological effects. Anti-malarial drugs were tested on *Salmonella Typhimurium*, likewise, anti-typhoid drugs on *Plasmodium berghei*.

Results: Artemether/Lumefantrine Malaria clearance rate was high, 75% single and 100% combined sensitive recorded. Mean decreased PCV of 37% to 25%, and initial body weight loss of 18g to 14.6g. The synergistic Typhoid drug effects on Mice, revealed 100% Ciprofloxacin sensitivity, initial increased mean body weight of 15.3g and total WBC count of 7,300/cm³ to decreased weight of 14.6g and WBC of 5,900/cm³.

Conclusion: A significant curative drug effect between Malaria/Typhoid infection revealed similar antibody production, Pharmacological effects of these drugs used was observed as strong evidence of single and combined drug effects for their vaccine production.

Key words; Drug Effect, Malaria, Typhoid, Vaccine Production

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I. Introduction

Malaria is the most important parasitic infectious disease in humans. *Plasmodium* species which is the causative agent of malaria equally infects mice, as revealed in the anti-malarial activity of the plant extracts in a mouse model of chloroquine-resistant *Plasmodium berghei* ANKA strain ([1]). Globally, 3.2 billion people in 97 countries and territories are at risk of being infected with malaria and developing the disease. There is an increasing rate of death associated with malaria disease. In 2014, one hundred and nineteen million cases of malaria infection cases occurred, causing five hundred and eighty four thousand deaths ([2]). The development of in-vivo antimalarial drug activity has become an essential tool in research to study different drug activities, the immunological, and hematological and biochemical effects of the drugs on the parasite. In-vivo anti-malarial drug activity has become an essential tool to study drug effects on plasmodium and its specific stages at which the drug activity and parasite suppression are most effective. Studying the changes that occur in the parasitized erythrocytes, identifying stage-specific anti-malarial drug activity, is really paramount in this in-vivo anti-malarial drug activity in the mice.

The greatest effort and time have been invested in cultivation of the erythrocytic stages of the *Plasmodium* life cycle, specifically in the most important and deadly of the human malarial parasites, *P. falciparum* ([2]). Trager and Jensen pioneered the development of a procedure for continuous in-vitro cultivation of the erythrocytic stages of the human malaria parasite ([3]). A modified Peter's 4-day suppressive test was used to evaluate the antimalarial activity of the plant extracts in a mouse model of chloroquine-resistant *Plasmodium berghei* ANKA strain ([1]). This suppressive test has therefore become an essential tool to characterize the specific drug effects on *Plasmodium* in-vivo in mice. Hence, studying the drug suppressive activity that occurs in the parasitized erythrocytes in-vivo in mice and identifying stage-specific proteins, packed cell volume (PCV), Parasite's blood film microscopic examination and drug activities etc improves the development of new drugs and vaccines against malaria. The changes that occur in the parasitized erythrocytes and identifying stage-

specific anti-malarial and Anti-typhoid drug activities of the *Plasmodium* parasite and *Salmonella* bacilli also produces a better knowledge of their drug effect. Enteric fever is a severe human disease usually caused by *Salmonella typhi* or one of the paratyphoid bacilli. Although the etiology of this important infectious disease has been understood for nearly a century, it has been only relatively recently that the host-parasite relationships involved in the expression of acquired resistance to enteric disease has become clearer ([4]). *Salmonella* is a pathogen of worldwide importance, causing disease in a vast range of hosts including humans. There are two species of *Salmonella*, *S. bongori* and *S. enterica*. *S. enterica* is comprised of six subspecies and over 2500 serovar ([5]). *S. Typhimurium* is a bacterial pathogen that poses a great threat to human and animals. Mice represent a small animal model for the study of the immune response to *S.typhi* and the development of vaccine against this important human pathogen ([5]).Mice that have ingested *S.Typhimurium* will come down with typhoid, its infection leads to seveal impairment on the mice's organ tissues such as the spleen and the ileum which are characterized by splenomegaly and edematous- villa respectively ([6]). In order to discover host response to *S.Typhimurium* infection, the infected mice blood was collected, for total white blood cell count (WBC Count). The stool of the mice will be collected and cultured in Desoxycholate Citrate Agar (DCA) media for isolation of the *Salmonella*. In response to systemic infection, mice usually present specific behaviors such as reduced activities just like in humans ([7]).Other serovar, *S. Typhimurium* strain 14028 and *Paratyphi C* strain BAA1715 were also found to be fully virulent in mice. The oral Lethal Dose (LD₅₀) for 14028 and BAA1715 were 4.5×10^5 and 1.6×10^5 CFU, respectively. Other strains of Paratyphi C have been shown to cause disease in mice ([8]). Furthermore, this study was performed in strict accordance with animal use protocols approved by The Institutional Animal Care and Use Committee (IACUC) Animal House Unit, Pharmacology Department Faculty of Pharmaceutical Sciences, University of Jos Nigeria, protocol number K109056570). In order to achieve a better knowledge of *Malaria* parasite and typhoid drug activities, which will be used in the development of new drugs and vaccines production against Malaria and Typhoid. Since enteric fever is a severe human disease, and similar consequences presenting the same pharmacological effects in mice, just as that caused by *Salmonella typhi* or one of the Paratyphoid bacilli. Therefore, Mice that have ingested *S.Typhimurium* will come down with typhoid, its infection leads to seveal impairment on the mice's organ tissues such as the spleen and the ileum which are characterized by splenomegaly and edematous- villa respectively. Hence, the urgent need for this in-vivo study in Mice model cannot be over emphasized.

1.1 Aim and objectives of the study

1.2 Aim

This research aimed to determine and assess the pharmacological in-vivo anti-malarial and anti-typhoid drug effects on *Plasmodium* and *Salmonella* Species in the Albino Mice.

1.3 Objectives

1. To determine the in-vivo activities of the anti-malarial and anti-typhoid drugs in the Albino Mice.
2. To determine the synergistic in-vivo drug effect of the anti-malarial and anti-typhoid drugs in the Albino Mice.
3. Setting up of tools and the assessment of pharmacological index as indicators for anti-malarial and anti-typhoid Vaccine production.

II. Materials and Method

2.1 Study Area/Location

The research was conducted in the Animal House unit of the Pharmacological Department Faculty of Pharmaceutical Sciences and in the Laboratory unit of the University Health Services (UHS), University of Jos Plateau State Nigeria. The animal house unit houses the experimental animals for researches, performed in strict accordance with animal use protocols. While the University clinic Laboratory UHS is located at the permanent site of the institution, along Bauchi road in Jos North local Government Area, Capital of Plateau State in Nigeria. Jos is the commercial strength of the state; Jos covers an area of about 9400km of the crystalline complex in the North-Central of Nigeria ([9]). The UHS laboratory was chosen because it is known to be the centre for Academic Research Excellence with précised quality laboratory tests.

2.2 Study Population

The Institutional animal care unit houses different animals used for research purposes including mice of different species. Their Albino Mice population aged six weeks was sixty, out of which thirty two was used. The UHS Clinic, have an estimated population of 8000 people patronizing it, as at the time this study was conducted.

2.3 Ethical Consideration

The Ethical Clearance was obtained and approved by The Institutional Animal Care and Use Committee (IACUC) Animal House Unit, Pharmacology Department Faculty of Pharmaceutical Sciences, University of Jos Nigeria, protocol number K109056570). In addition, the protocols for the study was reviewed and approved by Ethical Review Committee of Jos University Teaching Hospital (JUTH) Jos Nigeria.

III. Collection of Specimen

3.1 Sample Size Formula for Animal Studies

For animal studies there are two method of calculation of sample size. The most preferred method is the same method which has been mentioned in sample size calculation for testing the hypothesis above. While all efforts should be done to calculate the sample size by that method, sometimes it is not possible to get information related to the prerequisites needed for sample size calculation by *power* analysis like standard deviation, effect size etc. In that condition a second method can be used this is called as “resource equation method” ([10]). In this method a value E is calculated based on decided sample size. The value if E should lies within 10 to 20 for optimum sample size. If a value of E is less than 10 then more animal should be included and if it is more than 20 then sample size should be decreased.

$E = \text{Total number of animals} - \text{Total number of groups}$

In the experiment with animal study, were the researchers form 4 major groups of animal having 8 animals each for different interventions then total animals will be 32 (4×8). Hence E will be $E = 32 - 4 = 28$. This is more than 20 hence animals should be decreased in each group. So if researcher takes 5 mice in each group then E will be $E = 20 - 4 = 16$ E is 16 which lies within 10-20 hence five mice per group for four groups can be considered as appropriate sample size. This method was used because sample size calculation cannot be done by power analysis method explained above for testing the hypothesis.

3.2 In-vivo Culture of *Plasmodium Berghei* and *Salmonella Typhimurium* in the Albino mice.

The *Plasmodium berghei* was donated from the donor mice infected from Ahmadu Bello University Zaria, Animal house which is now being housed in University of Jos Animal house unit of the pharmacology department for research. . The donor mouse was used to infect the Albino mice used in this research by cutting off a small portion of the mice tail. Through the processing of pass aging the tail, to obtain about 0.5 ml of its blood, out of this blood volume was used to make a 1 in 10 dilution. Normal saline was used as the diluents. From this stock, 0.2ml was used to re-infect the test mice for the research. The *Salmonella* strains (*S. Typhimurium* strain) used was obtained from the Bacteriology Laboratory Unit of National Veterinary Research Institute (NVRI) Vom Jos. From the stocked agar slant, the *S. Typhimurium* were cultured with *Salmonella Shigella* Agar (SSA) and sub-cultured onto nutrient agar media in a petridish, it was incubated at 37°C, this culturally isolated *S. Typhimurium* was used to infect the test mice following the procedure in the In-vivo Drug Culture of mice with *S. Typhimurium*.

3.3 Allocation of the Sample Mice ;(from the population in the animal house,)

The Randomized control trials (RCT) were applied to allocate the mice into groups. The control and the treatment groups, This was carried out by blind folded person by picking the experimental animals (mice) to be used from the mice population in the animal house. A total of sixteen mice was picked from the sample size number calculated using the formulae above.

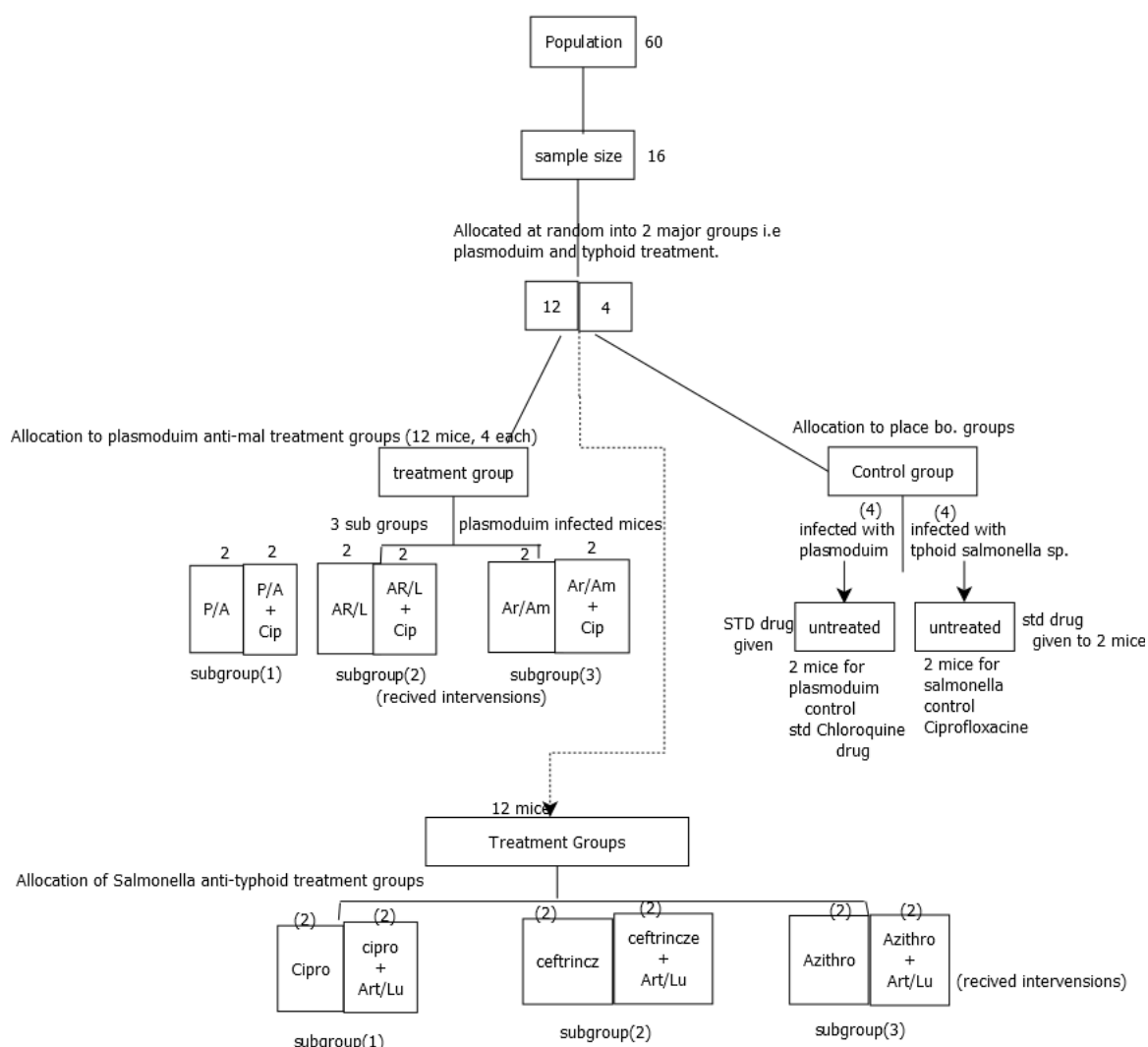
3.4 Procedural Allocation of the Selected Experimental Mice to their Groups

This also was done using Randomized control trial method to allocate the mice to groups. In this technique, a blind folded person picks the mice and allocates them to the groups meaning all participants have equal probability of being assigned to any group. Here, it was categorized into two (2) groups. The control and treatment groups, these mice were picked randomly into these groups. There was no bias because they are animals and it was so, in order to minimize biasness for proper assessment of the pharmacological drug effect and the drug synergistic effects appropriately.

After the allocation, the mice were assigned to a study group or treatment condition. A randomized control trial (RCT) therefore was used to assign the participating mice to treatment conditions at random. After infecting all mice randomly with the *Plasmodium berghei* [group (1) drug test] and *Salmonella Typhimurium* [group (2) drug test]. The *Plasmodium* infected group (1), their incubation period, or the duration for the mice to come down with malaria takes a period of 3days (72hrs) for the species used. While the incubation period for *Salmonella Typhimurium* used in group (2) was 8-10 days. The diagrammatic representation or sketch design of the mice, being assigned into two major groups, that is; the treatment and control groups are as shown below.

3.4.1 A Sketch/ Diagrammatic Representation of the Research Design

Diagram Showing The Research Design, Randomized Control Trial Method



(Sketch Diagram, Courtesy ([11])

3.4.2 Randomized Control Trial Design (RCT)

Allocation of the sixteen mice to the research groups: - the mice were allocated into **four sub groups**; Compromising of 4 mice in each Sub-group. Totaling 16 mice for the anti- malarial trial drug test. For these 4 Sub-group, three out of the four subgroups were subjected to 3 different anti-malarial and one anti-typhoid drug (Standard typhoid drug) that were administered to the mice intra-peritoneal (IP) in these groups. The fourth subgroup served as the control group, where no intervention or drug treatment was given to the trail animals.

IV. Laboratory Analysis and the In-vivo Drug Culture of mice with *S. Typhimurium*.

4.1 Mouse virulence, fecal shedding and In-vivo Culture

Male and Female mice (6 weeks old) obtained from Animal House Unit, were euthanized /infected with the *Salmonella Typhimurium* by incorporating it in their food, from the Overnight cultures of the *Salmonella* strain that was centrifuged at 5000×g and re-suspended in fresh peptone broth. The Mice was infected with 0.2 ml of the *Salmonella* strain (approximately 10⁹ totals CFU), Infection of mice with large oral doses of *S. typhi* induces an early septicemia in many of the animals ([4]). Then the dilution plating of each inoculums were used to determine the actual dose administered.

The Desoxycholate Citrate Agar (DCA) agar plates were used for the recovery of *Salmonella* from feces. Fecal pellets from surviving mice were homogenized and dilution plated for enumeration. The dilution of the anti-typhoid and anti-malarial drugs were prepared and administered to the grouped mice. Surviving mice were challenged with 10⁹ CFU of *S.Typhimurium* to assess if immunity was elicited by the test strain.

4.2 Procedure for the Mice Drug Trial Tests

In each group two of the mice were given one dose daily of the antimalarial drug (ie the required lethal dose) and the other two mice were given anti-malarial and anti-typhoid drug (that were used to check the synergistic effect of the drugs, also of the required lethal dose) as follows:

Sub-group 1 [House 1]: Artemether/ Lumefantrine only to the two mice, Artemether/ Lumefantrine and ciprofloxacin to the other two mice.

Sub-group 2 [House 2]: Artesunate Amodiaquine only to the two mice, Artesunate Amodiaquine and ciprofloxacin to the other two mice.

Sub-group 3 [House 3]: P-alaxin only to the two mice, P-alaxin and ciprofloxacin to the other two mice.

Sub-group 4 [House 4]: All the four mice served as the control groups no drug intervention was given to those mice in these groups.

Sub group 1, 2, 3 and 4 is the salmonella infected group mice.

The 16 mice in this sub-group received their treatment just like the malaria treatment as follows:

Sub group1 [House 1]: = ciprofloxacin only to two of the mice, Ciprofloxacin plus Artemether/Lumefantrine, to the other two mice.

Sub group 2 [House 2]: = cefuroxime only to the two of the mice, Cefuroxime plus Artemether Lumefantrine to the other two mice.

Sub group 6[House 3]: = Azithromycin only to the two of the mice, Azithromycin and Artemether/ Lumefantrine to the other mice.

The control / placebo group [House 4]: which will be subdivided into 2 sub-group one for *Plasmodium* control and the other sub-group for typhoid control will also house 2 mice in each of the sub-groups so that the first sub-group receives malarial standard control drug- Chloroquine while the second sub-group receives the typhoid standard control drug ciprofloxacin that was used in this research/study.

4.3 Lethal Dose (LD₅₀) determinations

Inoculums of 10000 *Salmonella Typhimurium* strain were prepared as described above. The suspensions were serially diluted in LB broth and groups of five mice were inoculated with doses ranging from 10¹ to 10⁹ CFU. The LD₅₀ was calculated using the method of Reed and Muench ([4]).

After the mice have received the interventions, the follow up commenced immediately for the malaria confirmed infected mice (confirmed by staining their thick blood films using field stain A and B). (Blood of the mice was collected by cutting a small part of the tail and pass aging the tail, a drop of blood (0.2ml) was used to make the thick blood film onto the glass slide that was examined and confirmed microscopically). The same methodology were applied, just like the ones in in-vitro culture and sensitivity for *Salmonella* isolation and Malaria parasite microscopy tests carried out in this study. The results obtained in this study are represented in the tables below.

V. Result

The results obtained from the in-vivo test of anti- malarial and anti-typhoid drugs in the albino mice are represented as follows in tables 1 and 2 below.

Table 1: Pharmacological effects of the single and combined drugs effect on Albino Mice

| Mice Mark House Pair Grp 1- 4 | Ave Result before infection Pharm. Index | | Ave.Mean BWt (gm) | Anti-Mal Drug Given Dosage. | | Ave Results After Treatment Pharm Index | | | |
|-------------------------------|--|-------------|-------------------|-----------------------------|--|---|--------------|---------|----------|
| | MP Seen (+) | Mean PCV(%) | | Pair grp Antima Drug | Mice Pair grp antimal/antisal drug given | MP Seen % + or - | Drug % sense | PCV (%) | BWt (gm) |
| Arm 1 | 2+ | 28 | 18.5 | P-ala, | P-ala+Cip | +25, | 75 | 23 | 23 |
| Head 2 | 3+ | 37 | 18.0 | A/L, | A/L + Cip | -75 | 100 | 25 | 25 |
| Tail 3 | 2+ | 31 | 17.3 | Art/A, | Art + Cip | +25, | 75 | 24 | 15.4 |
| Control4 | 3+ | 36 | 17.8 | No drug | No drug given | 100 | 100 | 26 | 17.6 |

Key; MP Seen (+) = Malaria parasite density/infestation positive seen; PCV (%) = Packed Cell Volume percentage; Ave BWt (gm) = Average Body weight in grams; Mice Pair grp antimal/antisal = Mice pair groups anti-malarial/ anti-*Salmonella* drug given; P- ala = P-alaxin; A/L = Artemether Lumefantrine; Art/A = Artesunate Amodiaquine.

Table 1: The result of the in-vivo drug effect on the albino mice shown in table 1 represents the four sub group marked houses; Arm1, Head2, Tail3, and control house 4. The Packed Cell Volume (PCV) and the mice body weight were taken and recorded before infecting them with the *Plasmodium Berghei*. After 3 days incubation period, the results of the load of malaria parasites seen, in the microscopic film examined were represented with the +ve and -ve signs before treatment with the antimalarial drugs as shown in the table 1. After the 3days course of treatment with the antimalarial drug and combination with Ciprofloxacin the standard antityphoid drug (No drug administered to the control mice in the control house 4) to evaluate the synergistic drug effects on *Plasmodium* species. The mice blood PCV, body weight and thick & thin films were retested and their value/results also recorded in the table 1.

Table 2: Assessing the synergetic effects of the commonly used anti-Salmonella and anti-Malaria drugs.

| Mice Mark House | Ave infection Pharm. Index | Result before | Mice Av. BWt | Anti Typhoid Drugs/ dosage x 5 days | | Ave Results After Infection Sal Typhi. M % | | | | | |
|-----------------|------------------------------------|---------------|--------------|-------------------------------------|------------------|--|----------------|------------------------|----------|----------|------|
| 1-4 | Mice Stool Cult (cm ³) | Tota WBC | | AntiSal drug | AntiSal+ antiMal | Gth on % sense | Gth on % sense | WBC (cm ³) | BWt (gm) | BWt (gm) | |
| Arm 1 | Sal Iso | 2,800 | 18.3 | Azi | Azi + A/L | 50 | 25 | 7400, | 15.6 | 7,400 | 15.6 |
| Head 2 | Sal Iso | 3,700 | 15,3 | Cip | Cip + A/L | 75, | 100 | 5900 | 14.6 | 5,900 | 14.6 |
| Tail 3 | Sal Iso | 3,100 | 16.4 | Cef | Cef=A/L | 50 | 25 | 7600 | 15.4 | 7,600 | 15.4 |
| Control 4 | Sal iso | 3,600 | 18,8 | No drug given | No druggiven | 100% | 100 | 5000 | 17.6 | 5,000 | 17.6 |

Key: *Salmonella* Isolates; Sal Iso, White blood cell; = WBC, Treatment x no of days given Drugs; = x 5 days; Culturally Isolated *Salmonella Typhimurium Percentage Growth on culture medium = Sal.Typhi.M*; % Gth on Cul M ; Pham Index = Pharmacological Index Assessed -Total white blood Cells counted ; presented/Transcribed into percentage: 0-2 mm, 3-4,5-6,7mm and above clearance of bacterial growth by 100%, 75%, 50% and 25% rate of drug sensitive respectively.. Sal. Typ. M % Growth on Culture = *Salmonella Typhimurium % Growth on Culture*

Table 2: The assessment result of the synergetic effects of the commonly used anti-Salmonella and anti-Malaria drugs were represented in the table 2. The experimental Albino mice were housed in the four sub group marked houses; Arm1, Head2, Tail3, and control house 4. The total White Blood Cell (WBC) and the mice body weight were taken and recorded before infecting them with the *Salmonella Typhimurium*, and are represented as *Salmonella* isolates before treatment. After 10 days incubation period, the stool produced by the mice that came down with typhoid were cultured and the isolated *Salmonella* bacteria represented as the isolates after treatment with the antityphoid drugs and their results are shown in the table 2. After the 4-days course of treatment with the antityphoid drugs and combination with Artemether/lumefantrin, the standard antimalarial drug, (No drug given to the control mice in the control house 4) to evaluate the synergistic drug effects on *Salmonella* species the experimental mice were retested. The mice's body weight were taken, blood for the WBC and Stool for culture, the value/results obtained were also recorded in the table 2.

VI. Discussion And Conclusion:

The results obtained from determining and assessing the pharmacological in-vivo anti-malarial and anti-typhoid drug effects on *Plasmodium* and *Salmonella* Species in the Albino Mice, revealed that *Plasmodium Berghei* and *Salmonella Typhimurium* initiated the infection of Malaria and Typhoid in the Albino mice and caused their disease conditions. The pharmacological curative effects of these drugs were assessed which resulted in the effective cure of the initiated Malarial and Typhoid in the Albino Mice.

The in-vivo activities of the anti-malarial and anti-typhoid drugs in the Albino Mice were effectively determined; this led to the prescription of Artemether Lumefantrin as the most effective curative antimalarial drug. Similarly, the Ciprofloxacin became the most effective antityphoid curative drug discovered in this study. The synergistic in-vivo drug effect of these anti-malarial and anti-typhoid drugs in the Albino Mice recorded very high synergistic drug curative effect presenting these two drugs as possessing unique broad spectrum synergistic effect on these two different organisms. Before and after the administration of these drugs, the weight of the mice was taken, their blood films, PCV, WBC, were also tested and the haematological parameters analyzed resulted to high significant values.

The relevance of these findings with regard to the development of an animal model for studying human typhoid fever vaccines and drug effectiveness were highly appreciated with these results obtained. Also worthy to note that together with the findings obtained from the in-vitro drug sensitivity tests, a revelation of a better understanding of drug effect and interaction of the pharmacological indexes were equally established. These are in line with the researches carried out by Philip and Frank (1974). Studying the drug suppressive activity that occurs in the parasitized erythrocytes in-vivo in mice and identifying stage-specific bacterial infections and antibodies produced as a result of these infections improves the development of new drugs and vaccines against Malaria and Typhoid fever.

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