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In Vitro and In Vivo Estimation of the Antibacterial and Antioxidant Activity of *Vernonia amygdalina* Leaf Extracts.

Olayinka Grace Okanlawon[1], Ruth Nzube Amaeze[2]
Zituade Seikegba Konboye[3], Sherifdeen Abiola Okunlola[4],
Saheed Adegbola Adeyanju [5], Alvan Chimere Okechukwu[6]

[1](Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Nigeria)
[2](Industrial Chemistry, Federal University of Technology, Owerri, Nigeria)
[3](Medical Laboratory Science, Niger Delta University, Nigeria)
[4](Microbiology, Obafemi Awolowo University, Nigeria)
[5](Bioinformatics, Teesside University, United Kingdom)
[6](Industrial Chemistry, Federal University of Technology, Owerri, Nigeria)

Abstract:
Background*: The antibacterial activity of Vernonia amygdalina leaf extracts was investigated in vitro and in-vivo against Salmonella Typhi (S. Typhi). The in-vitro susceptibility of S. Typhi was carried out using the agar well diffusion method. Twenty-five (25) rats separated into five groups were used for the in-vivo estimation, animals in the groups except the normal control group were gavaged orally with 9 X10^8 CFU/ml of S. Typhi and subsequently treated for seven days. The anti-typhoid activity was determined via in-vivo bacterial inhibition. Hematological parameters were assessed by an autoanalyzer. The biochemical parameters were determined by the spectrophotometric method. Histological analysis was performed using the Hematoxylin Eosin staining. Analysis of data was performed by one-way ANOVA and considered significant at $p<0.05$ using SPSS 21.0. V. amygdalina demonstrated no anti-salmonella activity in-vitro. V. amygdalina caused 90.31% inhibition of S. Typhi in-vivo. Treatment with the plant extract had no significant ($p<0.05$) effect on the hematological and biochemical parameters of rats. V. amygdalina showed antioxidant activity by causing a significant ($p<0.05$) decrease in MDA level. Histopathological changes observed in this study were not ameliorated by V. amygdalina. This study showed that V. amygdalina had moderate antityphoid but no protective activity on the liver and intestine.*

Key Word*: Antibacterial Activity; Antityphoid Activity; Antioxidant Activity; Salmonella Typhi; Vernonia Amygdalina;*

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I. Introduction

Salmonella Typhi is the cause of typhoid fever, usually referred to as typhoid. With an estimated incidence of 50 cases per year, it is the main cause of enteric illnesses among children in impoverished nations (Qamar, 2016). Typhoid is thought to cause 16 million illnesses and 6,000 000 deaths annually throughout the world (Appiah-korang, 2014). Weakness, abdominal pain, constipation, headaches, diarrhea, and vomiting are among the symptoms of the illness (Anna, 2014). These symptoms often appear six to thirty days after exposure and can range in severity from mild to severe (Ochiai, 2015). The initial infective dose, the virulence of the organism, and the host's immunological response all affect how severe the infection is (Adams, 1999). Eating or drinking food or water contaminated with the feces of an infected individual frequently results in the transmission of typhoid fever (Bhan, 2005). The preferred medication for treating typhoid fever at the moment is ciprofloxacin, but due to the widespread emergence of S. typhi that is resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole, other therapeutic options, such as the use of medicinal plants, are now being sought after (Girgis, 2009). The term "medicinal plants" refers to a variety of plants used in herbalism that have medicinal properties (Bassam, 2012). More than 3.3 billion people in less developed nations regularly use medicinal herbs, which are the "backbone" of traditional medicine (Davidson-Hunt, 2000). The chemicals found in medicinal plants are thought to be a rich source for the creation and synthesis of drugs (Lemma, 1991).

Furthermore, pharmaceutical intermediates (such diosgenin from Discorea sp.) and key medications like quinine, reserpine, and tinctures similar to Galenicals are all derived from medicinal plants (Lemma, 1991).
Asteraceae family member Vernonia amygdalina is a tiny shrub that grows in tropical Africa with elliptic-shaped, petiolate leaves that are approximately 6 mm in diameter (Figure 2.1). (Eloff, 2010). Because of its bitter flavor, it is frequently referred to as "bitter leaf." It has several names in Nigeria, including "Ewuro" in Yoruba, "Onugbu" in Igbo, "Oriwo" in Bini, "Ityuna" in Tiv, and "ChusarDoki or fatefate" in Hausa. In Cross River State, however, it is known as "Etidot" (Owoeye, 2010). Antinutritional elements such alkaloids, saponins, tannins, and glycosides are to blame for the bitter flavor (Tuah, 1995).
The leaves are a type of green leafy vegetable that can be eaten raw or cooked. Aqueous extracts of the leaves are also used as tonics to cure a variety of illnesses (Igile, 1995). Chimpanzees in the wild have been seen to consume the leaves when they have parasite diseases (Huffman, 2003). Many herbalists and traditional healers in Africa advise their patients to take its aqueous extracts as a remedy for a variety of illnesses, including emesis, nausea, diabetes, and loss of appetite. (Becker, 1983).

1.1.1 Botanical Classification

Vernonia amygdalina is scientifically grouped as belonging to the Kingdom Plantae. It is an angiosperm, of the order Asterales, of the family Asteraceae, genes Vernonia, and species *V. amygdalina.*

Figure 2.1 *V. amygdalina* collected from Isale general, Ogbomosho (17/02/2022)

1.1.2 Chemical composition of *V. amygladina*

From the leaves of Vernonia amygdalina, several researchers have isolated and described a variety of chemical compounds with strong biological activity. Sesquiterpene lactones (Cimanga, 2004), flavonoids including luteolin, luteolin 7-O-glucosides, and luteolin 7-O-glucuronide, steroid glycosides (Jisaka, 1992), and vernonioside A, B, A1, A2, A3, B2, B3, and A4 are some of the previously isolated compounds in Vernonia amygdalina Del. (Igile, 1995). Edotides from the plant's aqueous extract were described in (Cimanga, 2004). In a recent study, Owoeye et al. (2010) isolated and characterized a sesquiterpene lactone called epivernodalol, another elemanolide, from the dichloromethane fraction of Vernonia amygdalina. Previously, Koul et al. (2003) isolated the compound epivernodalol from Vernonia lasiopus, a different species of the plant. In Table 2.1, several of these compounds are listed.

1.1.3 Traditional uses of *V. amygdalina*

In Nigeria, this plant's leaves are used as a green vegetable or as a spice in soup, particularly the well-known bitter-leaf soup (Koul, 2003). It has been included in horse feed in Northern Nigeria as a tonic or fattening supplement known as ChusarDoki'in Hausa (Dalziel, 2002). Ethiopian tela beer has also been made using the leaves as hops (Getahun, 1999). The leaves are frequently used to treat fevers and are well-known in Nigeria and some other African nations as a quinine alternative (Abosi, 2003). The young leaves are used in traditional medicine as an expectorant, worm expeller, antihelmintic, laxative/purgative, antimalarial, and fertility inducer in subfertile women. It had been noted that some wild chimpanzees in Tanzania used this herb

to cure parasite-related illnesses (Huffman, 2003). In Figure 2.3, various traditional use of V. amygladina are shown.

Table 1.1. Bioactive compounds isolated from *V. amygladina*

Name of compounds	Class of compounds	Author(s)
Vernodalin	Sesquiterpene lactone	Kupchan*et al*,(1969)
Vernomygdin	Sesquiterpene lactone	Kupchan*et al*,(1969)
Vernoniosides	Steriod Glucosides	Jisaka*et al,* (1992)
A1,A2,A3,B1 Vernoniosides	Steriod Glucosides	Jisaka*et al,* (1992)
A4,B2,B3 Vernoniosides	Steriod Glycosides	Igile*et al*, (1995)
D and E Vernodalol	Sesquiterpene lactone	Ganjian*et al*, (1983); Erasto*etal*,(2006)
Epivernodalol	Sesquiterpene lactone	Owoeye*et al*, (2010)

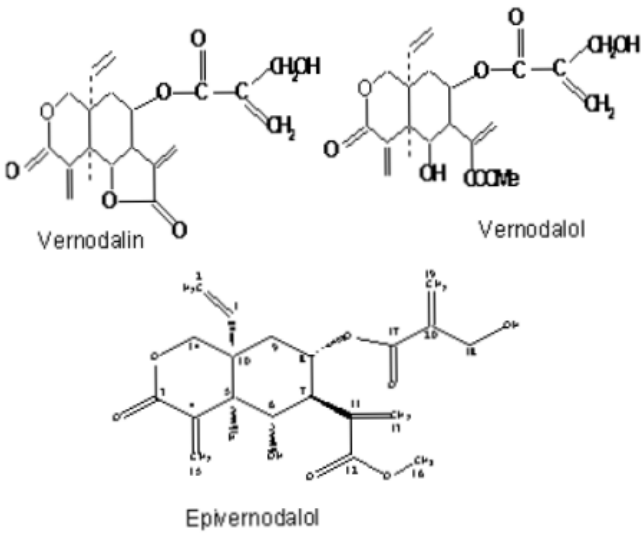

Figure 1.2 Some structures of compounds isolated from *V. amygladina*

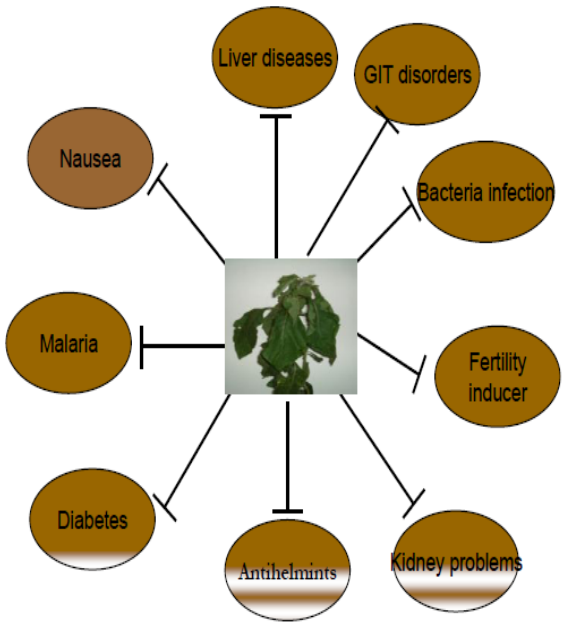

Figure 1.3Traditional uses of *V. amygladina* (Bitter leaf)

1.1.4 Bioactivities of *V. amygdalina*

1.1.4.1 Antimicrobial activity

According to Eleyinmi (2008), Anteridiacholerasius and Listeria monocytogenes were both inhibited by the methanolic extract of G. latifolium leaves. Only E. coli and P. aeruginosa demonstrated inhibitory action in the

aqueous extract. According to Nwinyi et al. (2008), the ethanolic leaf extract had a stronger inhibitory impact on Staphylococcus aereus and E. coli than the aqueous extract did. The zone of inhibition has a diameter of 6.0 to 10.0 cm. The essential oil from the leaves of G. latifolium and its aqueous and ethanolic extracts were tested for their ability to inhibit the growth of bacteria that were isolated from HIV patients in Lagos, Nigeria, including Staphylococcus species, E. coli, Shigella species, Salmonella species, Klebsiella pneumonia, Pseudomonas aeruginosa, and Onchrobactrumanthropi (Adeleye et al., 2011). The study's inhibitory effects were similar to those of ampicillin but were less potent than those of ciprofloxacin and chloramphenicol.

1.1.4.2 Antidiabetic activity

The anti-diabetic properties of G. latifolium's aqueous and methanolic extracts were proven by injecting the extracts intraperitoneally into diabetic rats produced by alloxan (Akah et al., 2011). Rats administered ethanolic and aqueous leaf extracts of G. latifolium showed a dose- and time-dependent drop in blood glucose levels as compared to the control group, according to Udo et al. (2013). Sylvester et al. (2015) found a significant (p0.05) decrease of blood glucose by 66.34% in experimental rats treated for streptozotocin-induced diabetes mellitus. Total cholesterol (TC) and LDL cholesterol (55.42 and 55.4%, respectively) increased in their study as a result of the diabetic induction. The plant extract treatments reduced TC by 58.70% and LDL by 71.70%.

1.1.4.3 Antioxidant activity

According to Nwanjo et al. (2006), the aqueous extract of G. latifolium leaves displayed anti-lipid peroxidase function. In their research, the extract considerably (p 0.05) boosted superoxide dismutase activity and decreased levels of malondialdehyde, a plasma lipid peroxidation product. Eze and Nwanguma reported on their investigation into the antioxidant activity of tannin extracts from the leaves of G. latifolium on partly purified lipoxygenase from seeds of Cucumeropsismonii (2013). The tannin fraction's inhibition of lipoxygenase was comparable to that of two well-known antioxidants, ascorbic acid and propyl gallate. According to Usoh and Akpan (2015), the antioxidant impact is increased when G. latifolium and Ocimumgratissimum leaves are combined.

1.1.4.4 Anticancer activity

In-vitro tests by Iweala et al. (2015) showed that G. latifolium leaf extract had potent inhibitory action against human lung cancer and human breast adenocarcinoma. Additionally, they in-vitro tested the extract's ability to scavenge free radicals against 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). According to Atangwho et al. (2009), phytochemicals' capacity to scavenge free radicals and act as antioxidants may help protect against cancer.

1.2 *Salmonella typhi* inthe Pathogenesis of typhoid fever

A gram-negative, rod-shaped facultative anaerobe that only affects humans, Salmonella typhi. Scientists are baffled as to why this disease has such a selective host behavior and does not infect other creatures (Midala et al, 2005). In order to move throughout the peripheral lymphatic system, including the bone marrow and Payer's patches, this bacterium first multiplies inside the human body's digestive tract (Brusch, 2001).

The organisms adhere to the small intestine's epithelial cells, pierce the submucosa, and then move through the lymphatics and into the bloodstream. The bacteria then cause temporary bacteremia and seed the kidneys, gall bladder, and reticuloendothelial system (liver, spleen, bone marrow). From the gallbladder, the organisms re-enter the gut, causing Peyer's patches, inflammation, and ulceration. The time of incubation ranges from 5 to 21 days (Kaur, 2012).

The initial infective dose, the virulence of the organism, and the host's immunological response all affect how severe the infection is (Adams, 1999).

1.3 Epidemiology of Typhoid fever

South Asia and sub-Saharan Africa are the two main regions where typhoid fever cases and fatalities occur among populations without access to drinkable water, proper sanitation, and sanitary facilities (Crump; Mintz, 2010). According to a report, typhoid fever causes an estimated 21.5 million illnesses and 200,000 fatalities worldwide each year. Out of an estimated 427 million people in Africa, 4.36 million cases are estimated, and Nigeria is one of the tropical nation’s most frequently affected by the disease (Zige et al., 2013). Enteric fever tends to be more prevalent in the tropics during the hot, dry seasons, when the concentration of bacteria in rivers and streams rises, or during the rainy season, when floods can spread sewage to sources of drinking water. The annual incidence of typhoid in some regions may reach 1,000 cases per 100,000 people. Typhoid is primarily a pediatric illness in these regions, and the main cause of the infection is S. typhi excretion in the stool both during and after the infection. S. typhi infections are often mild and self-limiting in these regions (Kaur, 2012).

1.4 Life cycle of *S. typhi*

Salmonella remains in a membrane-bound compartment known as the Salmonella-containing vacuole for the duration of its intracellular life (SCV). The SCV is most likely a distinct compartment created by the coordinated activity of numerous bacterial virulence factors (Figure 2.4). Salmonellae that are virulent can alter this vacuole to avoid being destroyed in the endocytic pathway and to multiply inside of host cells (Haraga et al., 2008). In a mouse model of typhoid fever, Salmonella's capacity to persist and multiply within host cells is directly related to its systemic pathogenicity. In a mouse animal model of typhoid fever, mutant strains with

auxotrophies that prevent intracellular reproduction are also less virulent (Fields et al., 1986). Salmonella may quickly multiply in a number of eukaryotic cell lines, but it seems to proliferate much more slowly in cells found in tissues of infected animals, indicating a more constrained environment in vivo (Mastroeni etal, 2009). Based on studies of bacterial reporter strains, microarray analysis, and the characteristics of auxotrophic strains, the SCV is frequently thought of be a nutritionally deficient environment. Salmonella's ability to reproduce inside the SCV, however, shows that it has successfully adapted to this intracellular habitat.

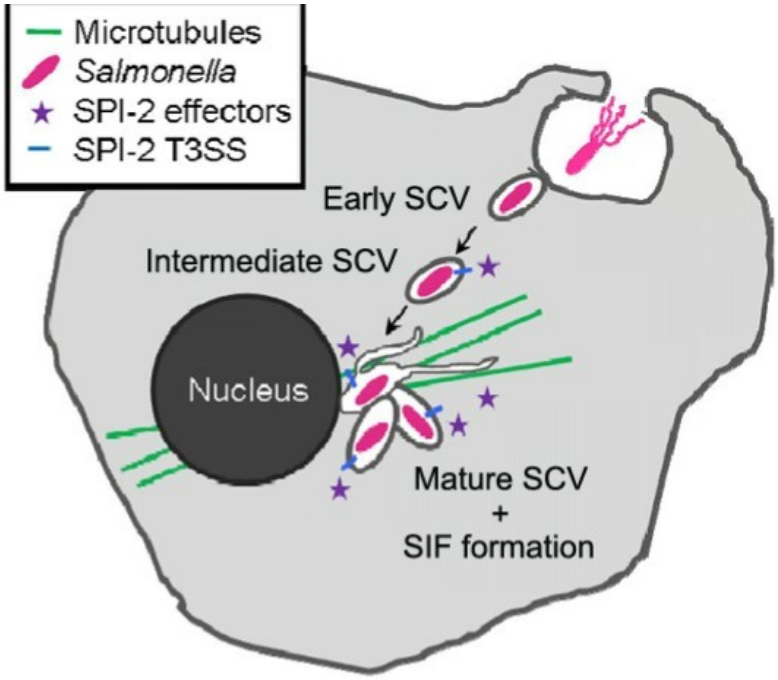

Figure 1.4 Intracellular lifecycle of *S. typhi* (TchawaYimga*et al.*, 2006).

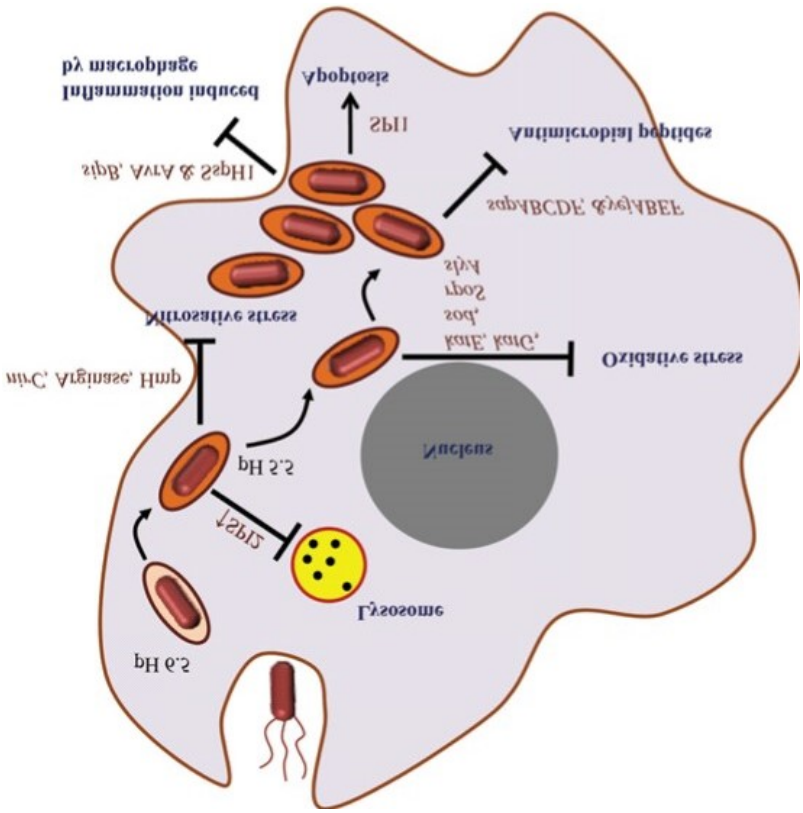

Figure 1.5 Immune evasion of *S. typhi* (Nizer, 2006).

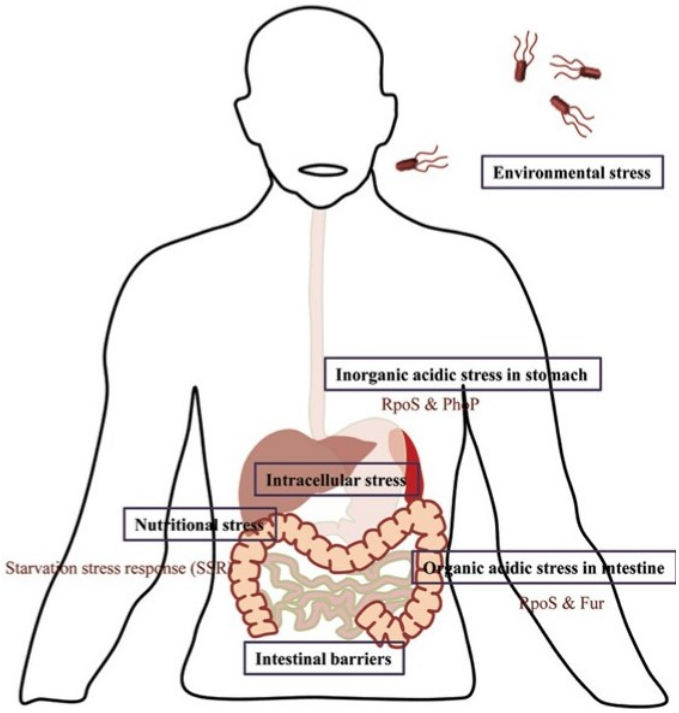

Figure 1.6 Challenges encountered by *S. typhi.* (Bearson, 1998).

1.5 Treatment of typhoid fever

Enteric fever is typically treated with antimicrobial monotherapy; however, it is unclear which medication is best and how long the treatment should last (Richard et al, 2007). In the USA, combination therapy with drugs like ceftriaxone/ciprofloxacin has also been widely employed (Crump, 2008). Fluoroquinolones were regarded as the medications of preference in the context of multiple-drug resistance (MDR) to first line agents (amoxicillin/ampicillin, cotrimoxazole, and chloramphenicol). However, parenteral therapy with a third-generation cephalosporin, such as ceftriaxone, or treatment with azithromycin have become increasingly widespread due to decreased drug susceptibility and complicated, fluoroquinolone resistance becoming widespread in endemic areas (Basnyat, 2010).

Fluoroquinolones and azithromycin have both been the subject of a Cochrane review for the treatment of typhoid in the past (Effa, 2011), however broad-spectrum beta-lactam drugs like third- and fourth-generation cephalosporins have not yet been comprehensively studied. Five to ten days for oral treatment with a fluoroquinolone or azithromycin, and seven to fourteen days for beta-lactams are advised as the risk of relapse may be higher with shorter beta-lactam therapy durations (Arora, 2011). Additionally, individual studies do not provide clear guidance on the recommended course of treatment or the relative advantages of different cephalosporins (Kalman, 1990). According to certain research, ceftriaxone may be superior to cefotaxime, while oral cefixime and ceftriaxone had comparable therapeutic efficacy. The intervention's description Beta-lactam molecules called cephalosporins have a 6-membered dihydrothiazine ring bonded to the betalactam ring, creating the cephem nucleus (Kalman, 1990). According to their antibacterial spectrum of activity, they have historically been split into four generations, with each generation often covering more Gram-negative bacteria at the expense of Gram-positive bacteria (Kalman, 1990).

Broad-spectrum 5th generation cephalosporins, like ceftaroline, have been created more recently, but they are not yet used routinely in clinical practice (Bazan, 2011). By attaching to penicillin-binding proteins, cephalosporins prevent the formation of cell walls and are bactericidal. They have a time-dependent killing activity that necessitates doses that are consistently higher than the pathogen's MIC. There are several dosage regimens, however some cephalosporins, like ceftriaxone, have the specific advantage of having a half-life that is long enough to be administered once daily (Kalman, 1990).

The extracellular fluid of the majority of tissues is well distributed by most cephalosporins, and some of the later-generation cephalosporins can sufficiently penetrate the cerebrospinal fluid to treat infections of the central nervous system. Cephalosporins do not however penetrate well into the intracellular compartment (Kalman, 1990). Most cephalosporins are eliminated through the renal system, however others, including ceftriaxone and cefoperazone, have considerable biliary excretion as a side effect (Kalman,1990). Although

some patients may exhibit hypersensitivity, they are typically well tolerated; other symptoms such interstitial nephritis and hepatic dysfunction may also be present (Kalman, 1990).

Enteric fever can be treated with third and fourth generation cephalosporins, which often have activity against salmonellae. There have been isolated reports of third-generation cephalosporin resistance in S. typhi isolates from Pakistan, Bangladesh, and the Philippines, as well as widespread reports of plasmid-mediated extended-spectrum cephalosporin resistance in non-typhoidal salmonellae (Paterson, 2006). (Al-Naiemi, 2008; Abdullah, 2012).

1.5.1 Mechanisms of antimicrobial resistance in *S. Typhi*

The following are some ways that Salmonella typhi resists the effects of antibiotics:

Reduced permeability of the antimicrobial agent, efflux or transport of the antimicrobial, modification of the antimicrobial target site, and inactivation of the antimicrobial agent are some of the processes that can occur.

Most medication resistance is genetic in nature, either from chromosomal mutations, plasmid or transposon acquisition, or both (Denyer et al., 2011).

The aim of this study is to evaluate the antimicrobial and anti-typhoid activity of *Vernonia amygdalina (bitter leaf)* against *Salmonella Typhi* in-vitro and in-vivo.

II. Material And Methods

2.1 Chemicals

Mueller Hinton agar, Mueller Hinton broth, Salmonella Shigella agar (SSA), 10% formalin, MDA (fortess, UK), GSH (fortess, UK), Albumin (fortress UK), Lactate dehydrogenase (fortess, UK) and Total protein (Randox) kit, phosphate buffered saline, ethanol, ciprofloxacin and chloramphenicol.

2.2 Methodology

2.2.1 Collection and identification of plant samples

Leaves of *V. amygladina* were collected from Isale general, Ogbomosho North Local Government Area of Oyo State, Nigeria, in February 2022. The plant was identified and authenticated by a taxonomist at the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomosho.

2.2.2 Preparation and extraction of plant samples

Prior to air drying, the plant materials were washed in water to remove dirt. The washed leaves were air-dried for 2weeks under careful watch. After air drying, the leaves were pulverized using electric blender. 100 g of ground sample was soaked in 1000 ml of distilled water in a conical flask and stored at 4^{o}C for 72 hours with regular shaking. At the end of the 72 hours, the sample was filtered over No 1 filter paper and the filtrate was concentrated with a freeze drier. The freeze-dried sample was refrigerated until use.

2.2.3 Preparation of Innoculum

S. typhi used in this study was a clinical isolate obtained from University of Ilorin, teaching Hospital. The isolates was then introduced into Muller Hinton broth and incubated for at 37^{o}C for multiplication.

2.2.4 Standardization of innoculum

Mc Farlard standard was made by mixing 0.005 ml of 1.75% barium chloride dehydrate and 99.5 ml of 1% sulphuric acid. Mixing the two compounds form a barium sulphate precipitate which causes turbidity in the solution. The solution was adjusted to 0.12 absorbance at 620 nm. Mc Farlard standard was compared visually to a suspension of *S. typhi* such that the inoculum contain X10^{8}. Normal saline was added if thesuspension *of S. typhi* was too turbid when compared with the standard.

2.3 Determination of in –vitro sensitivity of *S. typhi*

The prepared agar were allowed to cool and then poured into plates i.e. petri dish and allowed to solidify. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking through over the entire sterile agar surface. This procedure was repeated by swabbing two more times, rotating the plate approximately 60° each time to ensure an even distribution of Inoculums. As a final step, the rim of the agar was swabbed.

The cork borer was used to make three wells (8mm) in the already inoculated plate making sure that the holes were distant apart to avoid overlapping of the zones of inhibition. The wells in the different plate was filled with 50 µl of *V. amygladina* (100 mg/ml), ciprofloxacin (500 mg/ml), chloramphenicol (500 mg/ml) and water respectively

Contents of the petri dishes were given below:

Agar + bacteria + distilled water (in well)

Agar + bacteria + ciprofloxacin (in well)

Agar + bacteria + chloramphenicol (in well)

Agar + bacteria + bitter leaf extract (in well)

After this, the agar plates were incubated invertedly for 18 hours in an incubator at 37^{o}C. Zones of inhibition were observed after incubation and measured for those that have inhibition zones, (Cheesbrough, 2006).

2.4 Experimental animal

Twenty-five (25) male albino rats (80-100 g) were obtain from papa Ajisani Area, Ogbomosho, Oyo state. They were acclimatized for 2 weeks and were fed with standard animal feeds with access to clean water. Animals were maintained under normal laboratory condition of light, humidity and temperature. The handling of the animals was in compliance with the animal management and care and the general guidelines for animal experimentation.

2.4.1 Animal Grouping

The animals were broadly divided into five groups according to their body weight, five animals in a group. The grouping and treatment of animals is indicated in Table 3.1.

Table 2.1: Grouping of animals

Group	Treatment
A (Normal control)	Distilled water only
B(Untreated;Negative Control)	Infected + distilled water
C(Standard drug 1)	Infected+ Ciprofloxacin (100 mg/ml)
D(Standard drug 2)	Infected+ Chloramphenicol (100 mg/ml)
E	Infected+ *V. amygladina* (500 mg/ml)

Infected: Gavaged orally with *S. typhi*

2.5 Experimental Design

After acclimatizing the animals, they were all infected (except the normal control) with *S. typhi* that has been adjusted to 0.5 Mc Farland Standard containing about 10^8cfu/ml of bacterial. Blood samples were collected from the tails of animals on the third day (48 hours) and inoculated on SSA plates to establish infection. After infection was confirmed, extracts and drugs were then given to each group as indicated in Table 3.1. Animals were treated every day and continued every other day for seven days.

2.5.1 Infection of animals

Animal infections were carried out by inoculating rat per os (through the mouth) using a gavage needle with 1ml of saline containing 10^8cfu of *S. typhi*.

2.5.2 Establishment of infection

Blood samples of the infected animals were collected through their tails two (2) days after infection. The blood samples were cultured on Salmonella shigella agar plates and incubated for twenty-four (24) hours at 37ºC. Infected animals showed growth on agar plates after twenty-four (24) hours, establishing infection in animals. This procedures as repeated every 72hours and the number of colony was counted and recorded. The percentage inhibition was calculated in the animals using the formular;

$$\text{Percentage inhibition} = \frac{\text{initial bacterial load} - \text{final bacterial load}}{\text{Initial bacterial}} \times 100$$

2.5.3 Collection of Samples

The animals were fasted overnight after seven days of treatment and sacrificed via cervical dislocation. Blood samples were collected from the heart region of the animals into EDTA bottles for hematology tests, some blood samples were collected into plain bottles, allowed to clot and centrifuged at 4000 rpm for 15 minutes. The serum was pipetted and stored in a separate plain bottle for biochemical analysis. The liver and small intestine of the animals were harvested and prepared for relevant biochemical analysis.

2.5.4 Homogenate Preparation

10% w/v of liver was homogenized in phosphate buffer (0.05, pH 7.4). The homogenate was centrifuged at 3000 X g for 15 minutes at 4ºC, supernatant was collected and then processed for the relevant analysis. (Gyam*et al,* 1990).

2.6 Biochemical Analysis

2.6.1 Determination of Albumin concentration

Principle

The measurement of serum albumin was based on its quantitative binding to the indicator 3, 3, 5, 5-tetrabromo-m cresol sulphonepthalein (bromocresol green, BCG). The absorbance being directly proportional to the concentration of Albumin in sample (Granted *et al* 1987; Double *et al.* 1971).

Procedure

BCG concentrate was firstly diluted with 87 ml of distilled water. Test tubes were then labeled sample, standard and blank test tubes and into the test tubes labeled blank, 0.01 ml of distilled water was added, 0.01 ml of serum into test tube labeled sample and 0.01 ml standard into the standard test tubes. 3 ml of BCG was added to each test tube and mixed. The mixture was incubated for 5 mins at 25 ºC and the absorbance was measured for sample and standard against reagent blank at 578 nm wavelenght.

Calculation**:**

$$\text{Albumin conc. (g/l or g/dl} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{standard conc.}$$

A sample: Sample absorbance
A standard: Standard absorbance

2.6.2 Determination of Lactate dehydrogenase activity

Principle

This test is used for quantitative determination of Lactate dehydrogenase in serum and plasma. Lactate dehydrogenase catalyzes the conversion of pyruvate to Lactate and NADH is oxidized to NAD in the process. The rate of decrease in NADH is directly proportional to the Lactate dehydrogenase activity and is determined by measurement of the rate of absorbance change at 340 nm (Amador*etal* 1963; Tietz, 1976).

Pyruvate + NADH $+H^+$ L-Lactate + NAD^+

Procedures

Into the cuvette, 100 µl of pyruvate was pipetted and 20 µl of serum was added. It was mixed and allowed to incubate for 60 sec at 25^{o}C then 200 µl of NADH was added. It was mixed and the initial absorbance was read, a timer was started simultaneouly and readings after exactly 1, 2, 3 minutes were taken at 340 nm.

Calculation:

U/L = 9682*ΔA 340nm/min

ΔA: Change in absorbance

2.6.3 Determination of Total protein

Principle

Cupric ions, in alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex that can be measured spectrophotometrically (Tietz, 1995).

Procedure

The test tubes were first labeled sample, reagent blank and calibrator. 0.02 ml of distilled water was added to reagent black test tubes, 0.02 ml of standard calibrator was added to standard test tubes, 0.02 ml of serum was added to sample test tubes followed by addition of biuret reagent to each test tube. Each test tube was mixed, incubated for 30 minutes at 25^{o}C and the absorbance were measured against the reagent blank at 546 nm.

2.7 Determination of lipid peroxidation

2.7.1 Determination of lipid peroxidation

Malondialdehyde (MDA) an index of lipid peroxidation was determined using the method of Buege and Aust, (1978). 1.0 ml of sample was added to 2 ml of MDA reagent and boiled at 100^{o}C for 15 mins. The reaction mixture was then allowed to cool down. The flocculent materials were removed by centrifuging at 3000 rpm for 10 mins. The supernatant was removed and its absorption was read at 532 nm against a blank. MDA was calculated using the molar extinction coefficient for MDATBA-complex of $11.5*10^5 M^{-1}$ cm^{-1}

Concentration of MDA= ΔA x TV/ ε x SV

Where ΔA =change in absorbance, TV=Total Volume, SV=sample volume, ε =molar extinction.

2.7.2 Estimation of reduced glutathione

The reduced glutathione (GSH) content of liver tissue a**s** non-protein sulphyhydryls was estimated according to the method described by Sedlak and Lindsay, (1968). To the homogenate (1.4 ml), 0.6 ml of trichloroacetic acid (TCA)was added, and centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent and 3.0 ml of buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm against the reagent blank.

Concentration of GSH= ΔA x TV/ ε x SV

$\varepsilon = 1.34 \times 10^4 M^{-1} cm^{-1}$

Where: ΔA = change in absorbance, TV=total volume, SV= sample volume, ε = molar extinction

2.8 Histological Procedure

The histological analysis as performed using the method of Pearse, (1980). Organs were fixed to prevent autolysis and putrefaction followed dehydration by immersing them through ascending grade of alcohol. After dehydration, the tissues were passed through two changes of xylene for two hours in a process called clearing.

The tissues were impregnated and embedded with molten paraffin wax. The paraffin wax was then allowed to solidify. The block of tissues were trimmed and sectioned into a suitable size. The sections were mounthed and dewaxed using a glass slide. The slides were placed in descending grades of alcohol from absolute to 50% for 3 minutes in each percentaSe of alcohol. The sections were stained using routine Haematoxylin and Eosin (H&E).

2.9 Statistical Analysis

The data obtained were subjected to analysis of variance (ANOVA) and the Tukey test was used to separate the means. The level of significance was considered at $P<0.05$ using the Statistical Package for Social Science (SPSS) 21.0.

III. Result

3.1 In-vitro anti *S. typhi* activity of *Vernonia amygdalina*

The result in table 3.1 showed the effect of *V. amygdalina* on the inhibition of *S. typhi.* The aqueous plant extract showed no inhibition with ciprofloxacin showing a higher zone of inhibition compared to chloramphenicol.

Table 3. In-vitro sensitivity of *S. typhi* to *V. amygladina*

Sample	ZI (mm)
Vernonia amygdalina	NI
Ciprofloxacin	54 ± 1.78
Chloramphenicol	49 ± 1.0

NI= No inhibition

3.2 In-vivo antityphoidal activity of *V. amygladina*

3.2.1 Inhibition of *S. typhi* by *V. amygladina*

The result in table 4.2 showed the effect of *V. amygladina* on in-vivo inhibition of *S. typhi.* Aqueous extract of *V. amygladina* showed higher percentage inhibition at the seventh day of treatment closely related with ciprofloxacin. Chloramphenicol showed the least inhibition of *S. typhi*

Table 3.2 Effect of *V. amygdalina* on the inhibition of *S. typhi* in rats

	Sample	Percentage inhibition	
		Day 5	Day 7
A	*V. amygdalina*	80.86%	90.31%
B	Ciprofloxacin	6.97%	99.8%
C	Chloramphenicol	74.41%	79.34%

3. 2.2 Effects of *V. amygladina* on hematology parameters of *S. typhi* infected rats

Table 3.3 showed the effect of *V. amygladina* on the hematological parameters of typhoid rats. The results revealed an insignificant ($p < 0.005$) decrease in the WBC count of *V. amygladina* treated group when compared with the untreated group. No significant ($p < 0.005$) difference was observed between the extract treated group when compared with the control, ciprofloxacin and chloramphenicol treated groups.

No significant ($p < 0.005$) difference was also observed in the RBC count, Packed Cell Volume (PCV) and Hemoglobin (HGB) concentration across the groups. Only in the normal control and chloramphenicol treated group was platelet count significantly ($p < 0.005$) increased when compared with the untreated group.

Lymphocyte (LYM) count was significantly ($p < 0.005$) increased in the chloramphenicol treated group when compared with the untreated group.

Table 3.3 Effect of *V. amygdalina* on hematological parameters in *S. typhi* infected rats

TREATMENT GROUP	WBC ($X10^3$)/µL	RBC ($X10^6$)/µL	HGB (g/dL)	HCT (%)	PLT ($X10^3$)/µL	LYM%
V. amygdalina	7.85 ± 0.85[b]	4.95 ± 0.71[b]	10.87 ± 0.54[b]	40.73 ±. 34[b]	319. 00 ± 69.00[b]	80.03 ± 2.29[b]
Chloramphenicol	2.23 ± 0.25 [b]	1.86 ± 0.21[b]	10.07 ± 0.19[b]	36.86 ± 0.90[b]	411.89 ± 4.67[a]	85.17 ± 0.28[a]
Ciprofloxacin	7.53 ± 3.16[b]	6.82 ± 0.71[b]	9.43 0.64[b]	42.80 ± 1.21[b]	633.00 ± 14.41[b]	80.30 ± 3.00[b]
Normal control	8.30 ± 2.52 [b]	7.04 ± 0.57[b]	10.4 ± 1.01[b]	42.80 ± 2.98[b]	265.56 ± 10.25[a]	81.03 ± 9.36[b]
Untreated	13.00 ± 0. 39	8.08 ± 1.15	`12.5 ± 1.20	37.05 ± 1.94	109.65 ± 8.35	73.5 ± 0.2

Values were expressed as mean ± SD and considered significant at P value <0.05. [a]significant difference when compared with the untreated group, [b]insignificant difference when compared with the untreated group, [c]significant difference when compared with the control group, [d]significant difference when compared with Chloraphenicol treated group, [e]significant difference when compared with Ciprofloxacin treated group.

WBC: White Blood Cell;
HGB: Haemoglobin;
RBC: Red Blood Cell (Erythrocyte count);
HCT: Haematocrit;
PLT: Platelet count
LYM: Lymphocyte count

3.2.3 Effects of *V. amygladina* on serum and liver biochemical parameters of *S. typhi* infected rats

3.2.3.1 Effects of *V. amygladina* on serum biochemical parameters of *S. typhi* infected rats

Figures 4.1 and 4.2 respectively showed the effects of *V. amydalina*on the serum TP, Globulin and Lactate dehydrogenase (LDH) of *S. typhi* infected rats. The result showed no significant ($p < 0.005$) difference in the biochemical parameters of the rats when compared across groups.

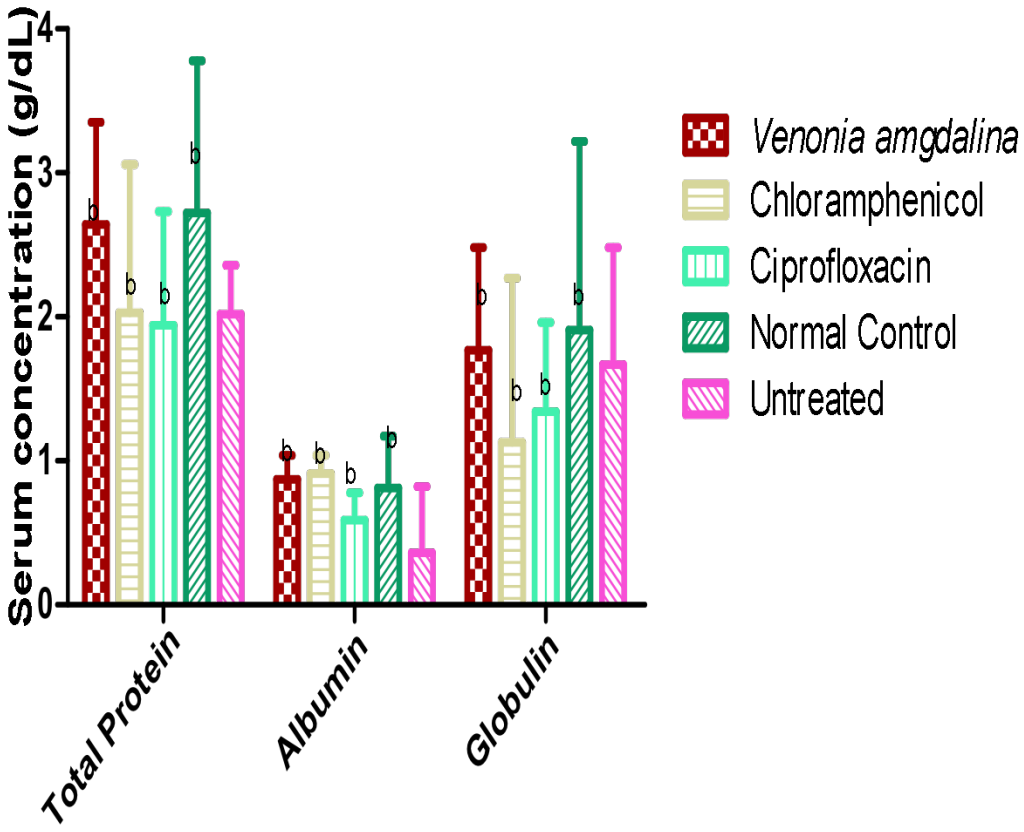

Figure 3.1 Effects of *V. amygladina* on serum biochemical parameters of *S. typhi* infected rats

Values were expressed as mean ± SD and considered significant at P value <0.05. [a]significant difference when compared with the untreated group, [b]insignificant difference when compared with the untreated group, [c]significant difference when compared with the control group, [d]significant difference when compared with Chloraphenicol treated group, [e]significant difference when compared with Ciprofloxacin treated group

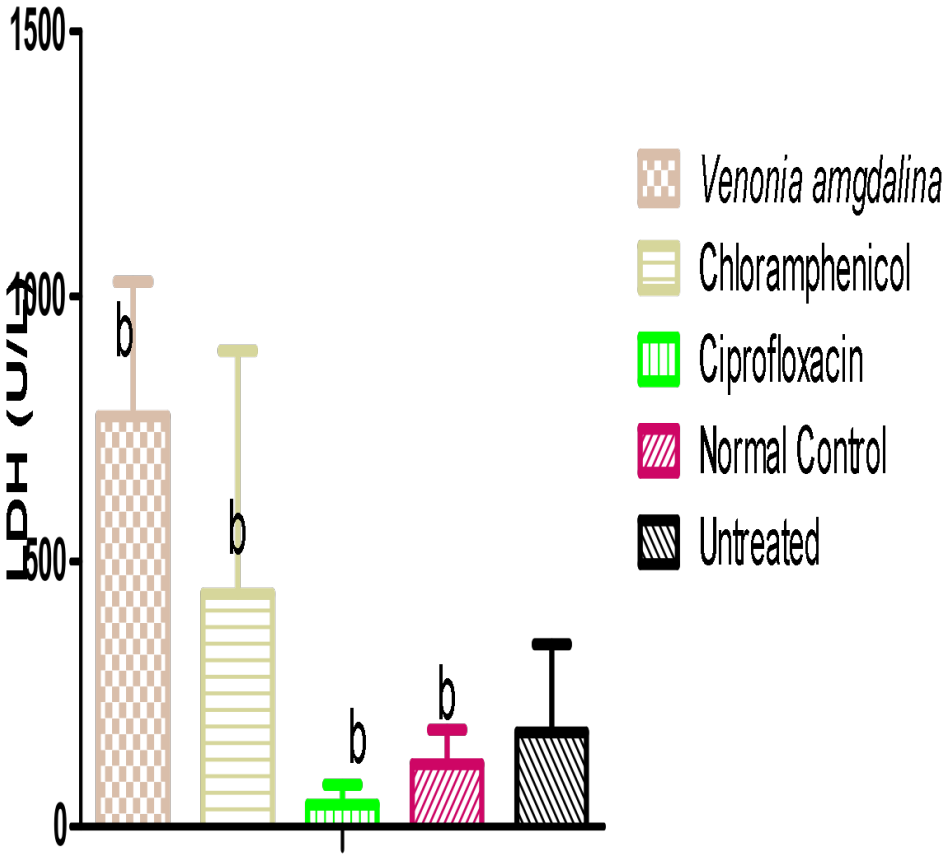

Figure 3.2 Effects of *V. amygladina* on serum biochemical parameters of *S. typhi* infected rats

Values were expressed as mean ± SD and considered significant at P value <0.05. [a]significant difference when compared with the untreated group, [b]insignificant difference when compared with the untreated group, [c]significant difference when compared with the control group, [d]significant difference when compared with Chloraphenicol treated group, [e]significant difference when compared with Ciprofloxacin treated group.

3.2.3.2 Effects of *V. amygladina* on liver biochemical parameters of *S. typhi* infected rats

Table 4.4and Figures 4.2 showed the effects of *V. amygladina* extract on the biochemical parameters of typhoid rats

The result showed no significant ($p < 0.005$) differnce in the liver total protein and globulin level when *V. amygladina* treated rats were compared with chloramphenicol, ciprofloxacin, normal and untreated groups. No significant ($p < 0.005$) difference was also observed in liver albumin level of *V. amygladina* treated rats, the normal control, chloramphenicol, and ciprofloxacin treated groups when compared with the untreated group.

Table 3.4 Effects of *V. amygladina* on liver biochemical parameters of *S. typhi* infected Rats

Group	Total protein (g/dl)	Alubumin (g/dl)	Globulin (g/dl)
Untreated	6.77 ± 0.89	0.17 ± 0.09	6.60 ± 0.93
Normal	6.02 ± 2.36[b]	O.18 ± 0.03[b]	5.84 ± 2.38[b]
Ciprofloxacin	6.64 ± 2.89[b]	0.20 ± 0.05[b]	6.44 ± 2.84[b]
V. amygladina	6.04 ± 0.78[b]	0.19 ± 0.07[b]	5.91 ± 0.71[b]

Values were expressed as mean ± SD and considered significant at P value <0.05. [a]significant difference when compared with the untreated group, [b]insignificant difference when compared with the untreated group, [c]significant difference when compared with the control group, [d]significant difference when compared with Chloramphenicol treated group, [e]significant difference when compared with Ciprofloxacin treated group.
Untreated: Infected with *S. typhi* and given distilled water

Normal: Given distilled water only
Ciprofloxacin: Infected with *S. typhi* and treated with ciprofloxacin
Chloramphenicol: Infected with *S. typhi* and treated with chloramphenicol
V. amygdalina : Infected with *S. typhi* and treated with *V. amygdalina*

3.3 Histology

3.3.1 Effect of *V. amygladina* on the liver histology of rats Infected with *S. typhi*

Results in 4.7 and 4.8 showed the representative photomicrograph of Hematoxylin and Eosin (H and E) stained liver section of rats infected with *S. typhi and* treated with V. *amygdalina.* The figure showed the portal triad (PT), hepatic vein (HPV), hepatic artery (HA) and the biliary duct (BD). The photomicrograph showed normal cytoarchitexture (Group A, C and D), degenerative cytoarchitexture (Group B) while group E showed mild to severe distorted morphological presentation characterized with fragmented hepatocytes, some fibrosis and heavy hemorrhage from the walls of the hepatic vessels (red arrows).

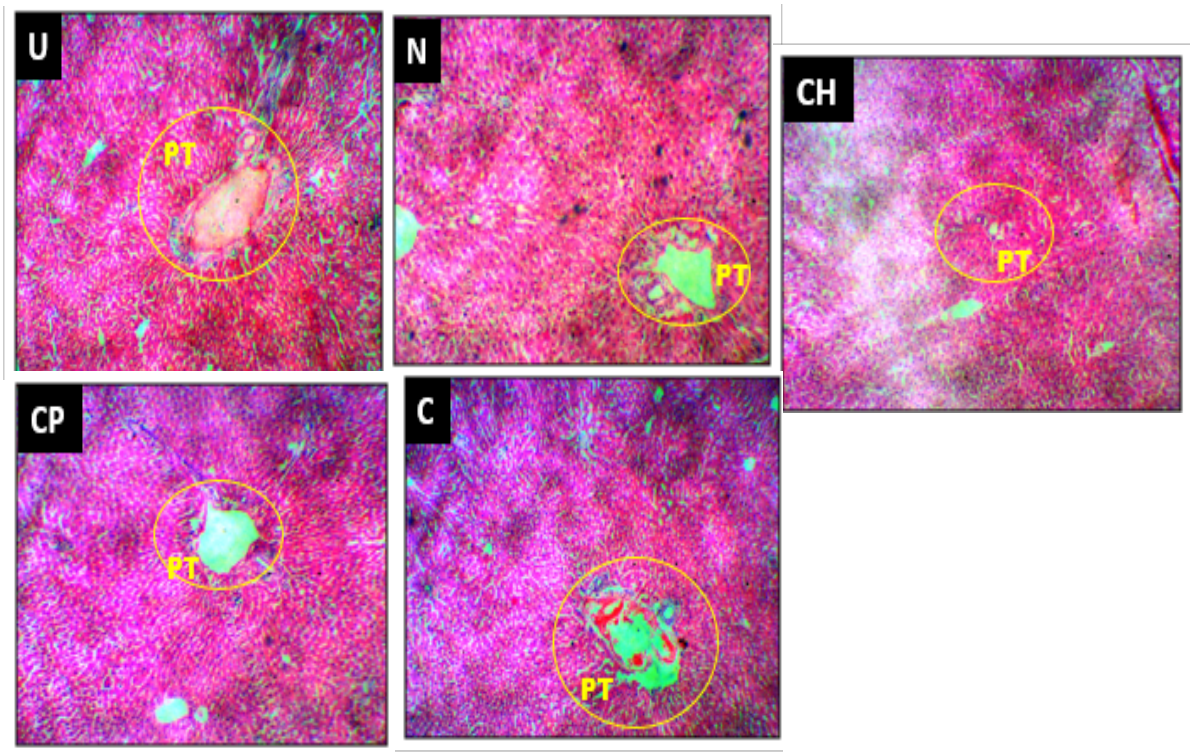

Figure 3.3 Liver representative photomicrograph of *S. typhi infected* rats treated with *V. amygdalina* MG X 40, H $ E stain.

A: Normal control – received only distilled water
B: Negative control – infected with *S.typhi* and given distilled water
C: Positive control – infected with *S. typhi* and treated with chloramphenicol
D: Positive control – infected with *S. typhi* and treated with ciprofloxacin
E: *V. amygladina* – infected with *S.typhi* and treated with *V. amygladina*

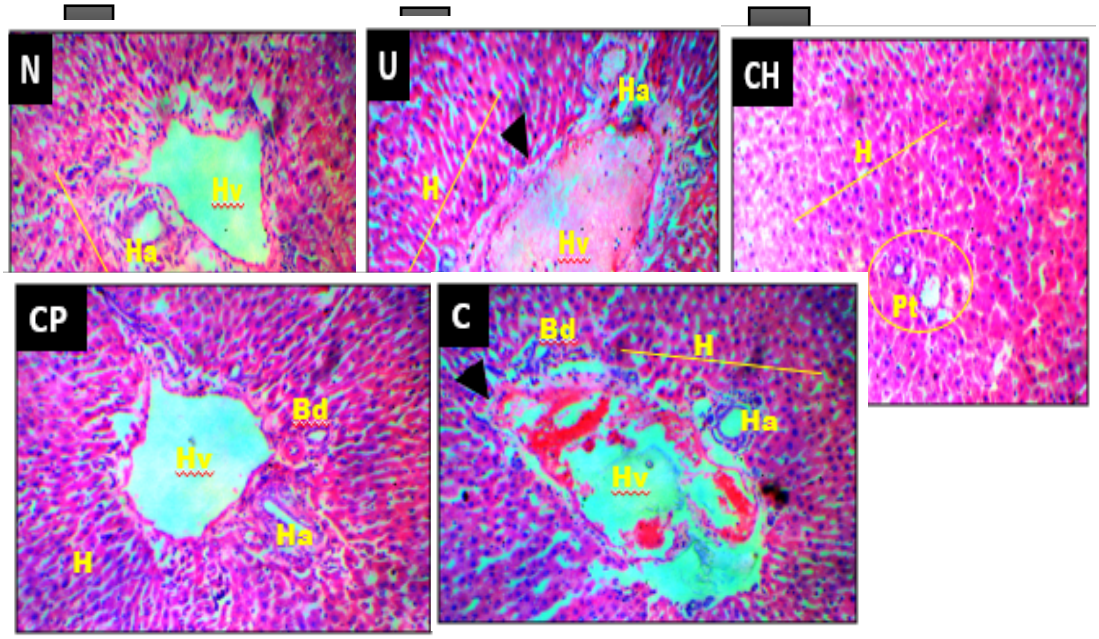

Figure 3.4 Liver representative photomicrograph of *S. typhi* infected rats treated with *V. amygdalina* MG X 100, H $ E stain
A: Normal control – received only distilled water
B: Negative control – infected with *S.typhi* and given distilled water
C: Positive control – infected with *S. typhi* and treated with chloramphenicol
D: Positive control – infected with *S. typhi* and treated with ciprofloxacin
E: *V. amygladina* – infected with *S.typhi* and treated with *V. amygladina*

3.3.2 Effect of *V. amygladina* on the intestine histology of rats Infected with *S. typhi*

Results in 4.7 and 4.8 showed the representative photomicrograph of Hematoxylin and Eosin (H and E) stained intestine section of rats infected with *S. typhi* and treated with *V. amygdalina.* The figure revealed the four layers of the gut: mucosa (M), submucosa (SM), muscularis (ME), lamina propria (LP), villi (V) and Brunner's glands (BG). Hypertrophy of the muscular layer is distinctly seen and indicated with (black and yellow Arrow head). B group showed more pathological presentation than other groups, while group E showed mild pathological presentation relative to group A, Group C and did not show appreciable Histopathological presentation

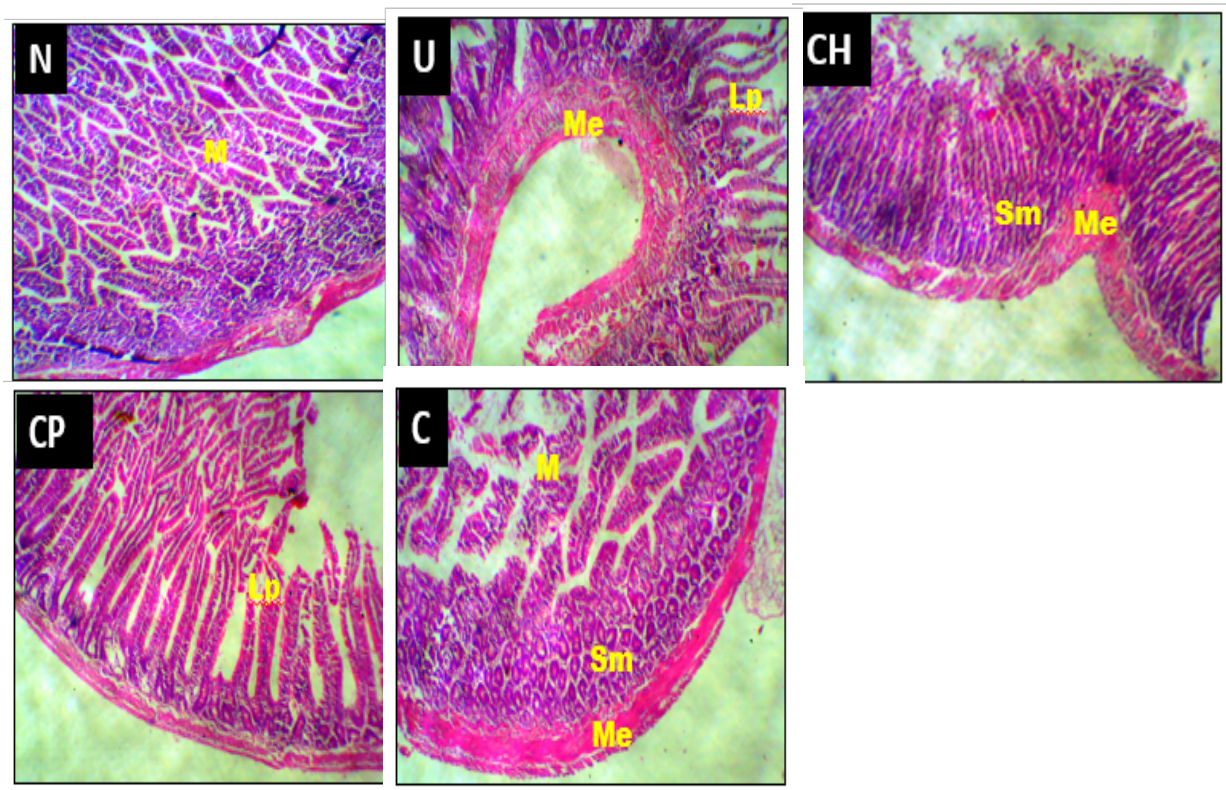

Figure 3.5 Representative photomicrograph intestine of *S.typhi*infected rats treated with *V. amygdalina* MG X 40, H $ E stain
A: Normal control – received only distilled water
B: Negative control – infected with *S.typhi* and given distilled water
C: Positive control – infected with *S. typhi* and treated with chloramphenicol
D: Positive control – infected with *S. typhi* and treated with ciprofloxacin
E: *V. amygladina* – infected with *S.typhi* and treated with *V. amygladin*

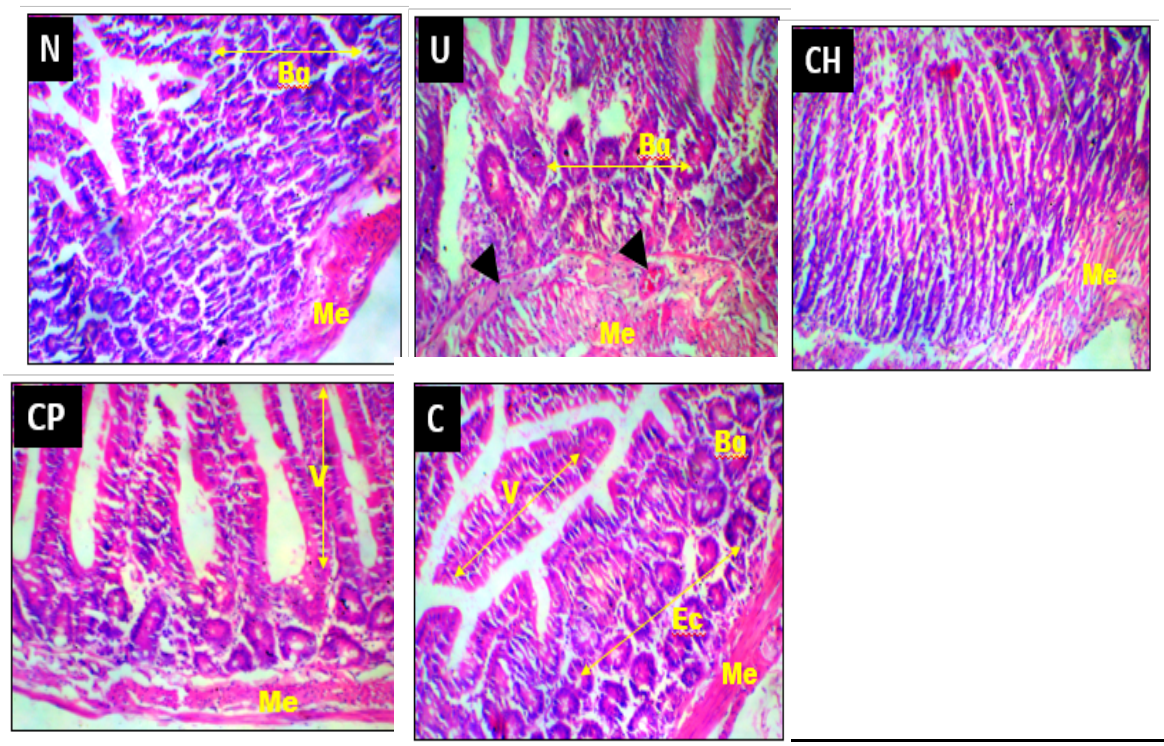

Figure 3.6 Representative photomicrograph intestine of *S. typhi infected* rats treated with *V. amygdalina* MG X 100, H $ E stain
A: Normal control – received only distilled water
B: Negative control – infected with *S.typhi* and given distilled water
C: Positive control – infected with *S. typhi* and treated with chloramphenicol
D: Positive control – infected with *S. typhi* and treated with ciprofloxacin
E: *V. amygladina* – infected with *S.typhi* and treated with *V. amygladina*

3.4 Antioxidant

Table 4.5 showed the effect of *V. amygladina* on the antioxidant parameters of *S. typhi* infected rats. The GSH level showed no significantly ($p<0.05$) difference across the group. The MDA concentration decreased significantly ($p<0.05$) in the treated group when compared with the untreated. In ciprofloxacin and chloramphenicol treated groups MDA concentration decreased significantly ($p<0.05$) when compared with the normal control. No significant ($p<0.05$) difference was observed in GSH concentration across the groups

Table 3.5 Effect of aqueous extract of *V. amygladina* on the antioxidant parameters of *S. typhi* infected rats

Antioxidant Parameters	GSH ($X10^4$/M/min/mg protein)	MDA ($X10^4$/M/min/mg protein)
Normal Control	0.28 ± 0.05^b	2.19 ± 0.00^a
Untreated	0.29 ± 0.01^b	4.62 ± 0.00
Ciprofloxacin	0.23 ± 0.02^b	$0.71\pm0.01^{a,c}$
Chloramphenicol	0.37 ± 0.05^b	$0.56\pm0.00^{a,c}$
V. amygladina	0.28 ± 0.05^b	1.26 ± 0.45^a

Values are Mean± S.E.M (n =5)
Values were expressed as mean ± SD and considered significant at P value <0.05. [a]significant difference when compared with the untreated group, [b]insignificant difference when compared with the untreated group, [c]significant difference when compared with the control group, [d]significant difference when compared with Chloramphenicol treated group, [e]significant difference when compared with Ciprofloxacin treated group.
Untreated: Infected with *S. typhi* and given distilled water
Normal: Given distilled water only
Ciprofloxacin: Infected with *S. typhi* and treated with ciprofloxacin
Chloramphenicol: Infected with *S. typhi* and treated with chloramphenicol
V. amygdalina: Infected with *S. typhi* and treated with *V. amygdalina*

IV. Conclusion

The bacterium S. typhi, which causes the potentially fatal sickness typhoid, is spread through consumption of tainted food or water (Bhan 2005). Typhoid fever is thought to cause 16 million bouts worldwide each year, resulting in 600,000 fatalities (Crumps, 2010). Typhoid fever is widespread in underdeveloped nations, and the vast majority of illnesses and fatalities there are related to peritonitis brought on by the perforation of ulcerated Peyer's patches in the small intestine (WHO, 1996). (Everest et al, 2001). To effectively battle the ongoing rise in typhoid fever morbidity and mortality, immediate intervention is required. Given their proven efficacy in treating a variety of ailments, plants are thought to be a possible candidate (Gasting, 2007). One of the plants that has historically been used to treat typhoid is V. amygdalina (Grover, 2004). This study assessed the aqueous leaf extract of V. amygdalina's anti-typhoid capability both in vitro and in vivo.In this study, *S. typhi showed* in-sensitivity to the aqueous extract of *V. amydalina*at the tested dose. It may be that the bacterial has developed resistance to show in-sensitivity to the plant extract. Previously, Ogbubile*et al.* (2007), Alo*et al,* (2012) reported inhibition of *S. typhi* by *V. amygdalina.* The contradiction in the observation may be due to differences in the extraction method.

In-vivo study was undertaken in a view to verify the therapeutic efficacy of *V. amydalina.* In this study, *V. amygdalina* showed comparable bacterial inbibition with ciprofloxacin. This is in agreement with the studies conducted by Salami *et al* (2013) who reported the sensitivity of *S. typhi* to aqueous extract of *V. amygladina* leaf. Several metabolites from plant species, including alkaloids, tannins and sterols, have previously been associated with antimicrobial activity (Chavasco, 2014). The detection of these classes of secondary metabolites in *V. amydalina* could explain the activity observed in this study.

Classically, during typhoid fever infection, there is leukopenia, a decrease in the number of circulating white blood cells, with eosinopenia and relative lymphocytosis (Weinberg *et al*. 2008). Plant extract could therefore fight against typhoid fever by inducing the proliferation of WBCs and lymphocytes in the body. Typhoid infection had no significant effect on the heamatopoietic activity of infected rats in this study. This observation is similar to the report of Lunga*et al*. (2014) where typhoid fever had no significant effect on the erythropoietic activity of typhoid rats.

Studies have showed that humoral immunity in typhoid fever is associated with increased serum globulin and total protein decreased serum albumin is associated with inflammation (Abro, 2009). As previously described by Bernaedi (2014), Emeuga*et al* (2014), Reinoso, (1998) and Amen *et al* (2012), typhoid fever caused significant increase in globulin level and total protein level but a decrease in albumin level.

In this study, no significant changes were observed in the biochemical parameters of typhoid rats except in serum albumin level of rats. This observation contradicts the report of Sameera *et al,* (2013) carried out on human. It may be that prolong infection is what caused some of the observed changes.

Malondialdehyde (MDA) is a good indicator of the degree of lipid peroxidation related to salmonella-induced tissue damage (Sharida, 2013). It is one of the end products in the oxidative breakdown of polyunsaturated fatty acids, and is a marker used frequently to evaluate lipid peroxidation in tissues (Lim, 2013) In this present study, the salmonella infection induced lipid peroxidation, suggested increased oxidative stress. This could be due to increased free radical formation accompanied by reduced antioxidant enzyme and antioxidant activities (Lim, 2013). Amelioration of lipid peroxidation by *V. amygdalina* is an indication of its antioxidant potential.

Hepatomegaly and moderate elevation of transaminase levels are the common findings that occur in typhoid fever (khosla, 1988). The pathophysiological mechanism by which Salmonella produces hepatic dysfunction, although not fully known as yet, is postulated to be either due to direct invasion or by endotoxemia with immune-mediated liver damage (Morgatern, 1991). In this study, *V amygdalina* was unable to protect the liver against *S. typhi* induced damage.

Intestinal perforation is a serious complication of typhoid fever (Bhaatta, 2006) that leads to high morbidity and mortality (Ugwu*et al.,* 2005).The high incidence of perforation in most developing countries has been attributed to late diagnosis and the emergence of multidrug resistant and virulent strains of *S. typhi* (Otegboya*et al,* 2002). The hypertrophy of the muscular layer was distinctly seen in the untreated and *V. amygdalina* treated groups. Observation in this study showed that *V. amygdalina* had moderate anti-typhoid activity but could not protect against organ complications related with typhoid fever.
tin 20mg.

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